UNIVERSITY OF WINCHESTER

The post-feeding larval dispersal of forensically important UK blow flies

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Doctor of Philosophy

August 2018

This Thesis has been completed as a requirement for a postgraduate research degree of the University of Winchester.

No portion of the work referred to in the Thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

I confirm that this Thesis is entirely my own work

Acknowledgements

Firstly, I would like to thank my supervisors Dr Martin Hall, Dr Amoret Whitaker and Dr Keith Wilkinson for their continued support and encouragement. Throughout the course of this PhD I have realised more and more how lucky I have been to have had such a great supervisory team.

I would like to thank Dr Sarah Arnold and the University of Greenwich for allowing me use of their servosphere and providing continued advice throughout my stay.

I am very grateful to Dr Andrew Hart and Dr Daniel Martín-Vega for their help with my thesis and general career.

Many thanks to Krzysztof Szpila for accepting me onto his annual forensically important Diptera identification workshop, in Poland. It was an intense, but highly interesting week, thank you so much to you and Andrzej Grzywacz for your patience.

I am extremely fortunate to have had such a great support system of colleagues, family and friends. I would especially like to thank Mr Andrew Mactaggart and Miss Sofia von der Schulenburg for their advice and endless patience.

Finally, I would like to thank the Royal Entomological Society for awarding me two CPF grants, the University of Winchester for giving me an 175th anniversary studentship and the Natural History Museum for making this PhD possible. I have thoroughly enjoyed every minute of it.

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ABSTRACT

The post-feeding larval dispersal of forensically important UK blow flies

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The post-feeding larval dispersal stage of forensically important UK blow flies is generally underrepresented in the literature. However, in order to give an accurate estimation of the minimum post mortem interval it is necessary to locate the oldest entomological specimens associated with the body. Often these are not on the body but have dispersed, consequently it is essential to determine how far from the body to search for post-feeding larvae, puparia and/or empty puparial cases and also how deep in the substrate to excavate. Therefore, this study examined the horizontal and vertical dispersal of post-feeding larvae, how dispersal is affected by different dispersal substrates, and whether post-feeding larvae, and the subsequent puparia, exhibit aggregation behaviour.

This study determined that substrate had a significant effect on the horizontal distance dispersed by post-feeding larvae. The majority (> 50 %) of *Calliphora vicina, C. vomitoria* and *Lucilia sericata* larvae dispersed less than 4 m if the dispersal substrate allowed larval penetration, such as soil, whereas an even, impenetrable surface (e.g. smooth plastic) forced the larvae to disperse the full length of the experimental apparatus, producing concentrations of puparia recovered from either end of the apparatus (i.e. where contact with the apparatus was greatest). In contrast, *Protophormia terraenovae*, a species not generally known to disperse far from the feeding source, were recovered within 50 cm of the origin in all but one of the experimental runs, in which the larvae dispersed over 5 m. This result shows that under certain circumstances *P. terraenovae* is capable of dispersing much further than has been reported in the literature.

In experiments that examined vertical dispersal, *C. vicina* larvae were capable of successfully dispersing up to 56 cm deep in the substrate where horizontal dispersal was impeded, and over 75 % of puparia were recovered within the top 20 cm of the substrate. However, if horizontal dispersal was possible then over 80 % of puparia were recovered from just the top 5 cm of the substrate.

Under most conditions, larvae appeared to aggregate prior to pupariation, and this was more defined when an impenetrable, smooth plastic substrate was utilised, resulting in puparia being recovered only from either end of the experimental apparatus, clumped together.

This study also reports the novel use of a servosphere to examine apodous insects. A servosphere was used to record the speed of *C. vicina* and *P. terraenovae* post-feeding larvae on a smooth plastic surface over time. During 5 minute runs the speed of both species remained almost constant over 4 days and *Calliphora vicina* larvae were approximately twice as fast as *P. terraenovae* larvae. However, during one experiment that tracked the changes in speed of *C. vicina* over one hour, speed initially decreased at a rate that reduced over time, until speed was almost constant for the final 20 minutes of the experiment. The servosphere was also used to examine phototaxis of *C. vicina* post-feeding larvae and, as expected, a negative phototactic response was recorded, within 5 seconds of the introduction of a light source.

The data produced during this study was consolidated to produce an addition to the current guides on collecting and preserving entomological evidence. This could be used by forensic entomologists, SOCOs and CSIs at crime scenes to optimise the collection effort.

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Chapter 1: Introduction

This chapter outlines the context of this PhD thesis in relation to the field of forensic entomology, while also explaining the structure of the thesis. The research outlined in this thesis examines the post-feeding larval dispersal of forensically important UK blow flies. To enable an understanding of the importance of any advances in the field, it is important to first appreciate the field of forensic entomology as a whole. This chapter provides a broad background of the field of forensic entomology, including a brief history of the subject, a short introduction to the biology of blow flies, followed by the challenges facing current practices and research in forensic entomology. A review of the published literature concerning this subject is necessary to recognise where the inconsistencies and gaps in knowledge exist and therefore to appreciate where this research fits in. The literature review (Section 1.5) is divided into sections that address different factors that may affect, and are affected by, the post-feeding larval dispersal of blow flies. Section 1.5.1 is the literature reviews of the factors that were examined during the experiments outlined in this study (Chapters 3 to 5). Sections 1.5.2 to 1.5.6 are literature reviews of the other factors that affect and are affected by post-feeding larval dispersal of blow flies that, due to time constraints, were not examined during this study.

1.1 Background

Entomology describes the study of insects and forensic entomology is the study of insects in a legal context (Byrd and Castner, 2009). There are an estimated 5.5 million species of insects worldwide (Stork, 2018), with an estimated 100,000 species of Diptera worldwide (Smith, 1986; Grimaldi and Engel, 2005); Diptera or true flies defines the order of the class Insecta that includes mosquitos, hoverflies and blow flies. It is estimated that there are 1000 species of the family Calliphoridae (blow flies) worldwide (Smith, 1986; Rognes, 1991), with roughly 30 species in the UK (Erzinçlioğlu, 1996). *Calliphora vicina, C. vomitoria, Lucilia sericata* and *Protophormia terraenovae* are the most common Calliphoridae species of forensic importance in the UK (Byrd and Castner, 2009).

The decomposition of a large animal creates a unique habitat that can be utilised by many different species including opportunistic feeders such as vertebrate scavengers (Amendt *et al.*, 2004; Byrd and Castner, 2009). After the death of an organism the bacteria that lived within the body immediately begin to rapidly reproduce, producing gaseous and liquid products. These olfactory cues attract certain insects that then proceed to colonise the organism (Burkepile *et al.*,

2006; Tomberlin *et al.*, 2011). Often blow flies are the primary colonisers of a corpse (Smith, 1986; Hall, 2007; Amendt *et al.*, 2011). The blow fly larvae themselves attract predators, parasites and parasitoids (Payne, 1965; Amendt *et al.*, 2004; Byrd and Castner, 2009; Cammack *et al.*, 2010). As the condition of the decomposing organism changes throughout the decomposition process, different insect species are attracted to the organism resulting in a wave of succession which utilises the decomposition of the organism from fresh to skeletal, recycling energy and nutrients (Amendt *et al.*, 2004; Byrd and Castner, 2009; Tomberlin *et al.*, 2011). This process is discussed in depth in the detailed overview of forensic entomology presented in Section 1.3.

The abiotic factors acting on the decomposing organism are also important and can produce significant differences in the decomposition process itself as well as affecting the different species that are attracted to the decomposing organism (Tomberlin *et al.*, 2011). Understanding the biotic and abiotic interactions of the decomposition of an organism is essential in understanding forensic entomology and the process of decomposition.

Forensic entomology is the study of insects in a legal context. The field of forensic entomology covers a broad range of subjects, typically divided into three disciplines: medicolegal, urban and stored product entomology (Byrd and Castner, 2009). This study focuses on the medicolegal branch. The most common and arguably the most important use of medicolegal forensic entomology is in the determination of the minimum post-mortem interval (minPMI) of a corpse (Amendt *et al.*, 2004). There are many methods employed by pathologists and other professionals to determine minPMI, such as the analysis of rigor mortis, rectal temperature and lividity (Hensge and Madea 2004; Dolinak *et al.*, 2005). However, the inaccuracies of these methods increase with time and for the period of 48-72 hours after death the entomological evidence is generally regarded as being the most accurate tool for determining minPMI (Amendt *et al.*, 2004).

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1.2 A brief history of forensic entomology

Using forensic entomology to assist in criminal investigations is not a new concept and was first documented in China in 1247. During a murder investigation, Song Ci – a death investigator – asked all of the suspects to present their sickles in the hot midday sun (McKnight, 1981). Due to the invisible traces of blood still present on the tool, blow flies were attracted to the murder weapon so when confronted with the evidence, the owner of the sickle confessed to murder. This case study shows a surprisingly advanced understanding of the relationship

between insects and decomposition. A comparative understanding of forensic entomology did not occur in the West until 1668 when Francesco Redi disproved the theory of spontaneous generation (Ramos-e-Silva, 1998). Prior to Redi's experiments, it was universally accepted that the larvae associated with the decomposition of carrion were generated from the decomposing substrate itself. Redi demonstrated that this was not the case, but that in fact the adult flies were attracted to the decomposing substrate, laid eggs and the larvae developed on the tissue, rather than being synthesised from it. Redi's discovery facilitated the study of forensic entomology, as insects were recognised as an external consequence rather than an internal synthesis of the decomposition process. Jean Pierre Mégnin published two books which outline the species of insects found during the insect succession on a corpse: *The Fauna of Tombs* (1887) and *The Fauna of Cadavers in Forensic Entomology* (1894). Their publication is widely regarded as marking the foundation of modern forensic entomology.

As the study of forensic entomology progressed, the research enabled the practice to be used in a legal context. In 1855, Dr Louis François Étienne Bergeret, a French physician, presented the first case report to include an estimation of minPMI (Bergeret, 1855). He estimated the minPMI of the body of a baby based on the insect evidence present and his previous experience of insects on dead bodies. Sarcophagidae and Lepidoptera were recovered from the mummified remains of a baby that was discovered in the chimney of a flat when renovations were taking place and he therefore concluded that the baby had died years earlier, exonerating the then current occupiers and leading to the conviction of the previous tenants (Bergeret, 1855). The first use of forensic entomology in a legal context in Italy occurred in 1874 when the entomologist Lazzaretti gave an estimation of minPMI after he examined puparia recovered from a decomposed corpse found in an attic in Padua (Lambiase and Gemmellaro, 2015). In Germany, entomological evidence was first used in court in 1889, when suspected evidence of trauma on the body of a baby was shown to have been caused by insect activity (Benecke, 2001). The father of the baby was suspected to have forced the child to drink Sulphuric acid due to the abrasions on the face and mouth of the baby (Benecke, 2001). These abrasions were determined to have been made by cockroaches therefore exonerating the father (Benecke, 2001). The first published case report that employed forensic entomology in Poland was in 1902. Stefan von Horoszkiewicz performed an autopsy on a child and after completing an experiment using cockroaches and pig skin, determined that the abrasions on the skin of the child were due to cockroach activity, not malintent (Bajerlein *et al.*, 2015). The first criminal case that used forensic entomology in Australia occurred in 1923; the insect evidence recovered from a corpse recovered from a river suggested that the infestation had occurred previously in a forest (Morgan, 2012).

It was not until 1935 that the use of forensic entomology was first documented in the UK to assist in an investigation (Hall, 2007). Larvae of *Calliphora vicina* were found and identified by Dr A. G. Mearns on the decaying remains of two dismembered bodies at a deposition site in Scotland. The larvae were aged and the minPMI estimation lead the police to the identification of the victims, as the estimated time of death corresponded to the date of the victim's disappearance. The estimation of minPMI, together with other forensic evidence, this led to the conviction of Dr Buck Ruxton for the murder of his common law wife and her maid (Smith, 1986; Hall *et al.*, 2015).

In 1947 the first criminal case that used forensic entomology in Belgium occurred when Marcel Leclerq, a medical student interested in flies, concluded that the Dipteran eggs recovered from the corpse of a child had been oviposited soon after the child had been reported missing (Dekeirsschieter *et al.*, 2013). In 1954 forensic entomology was first used in a criminal case in Spain to determine an estimation of minPMI on a decapitated body that was discovered in Madrid's main train station (Gómez-Gómez *et al.*, 2007). The first use of forensic entomology evidence in court in Canada was in the 1960s (Anderson, 2001), while a forensic entomologist first testified in court in South Africa in 1966 (Williams and Villet, 2006). In North America the entomologist Lamar Meek conducted forensic entomological research and testified as an expert witness in court during the 1970s (Wallace *et al*., 2015).

Forensic entomology has now become a widely respected discipline and is often relied upon to give estimations of minPMI in criminal investigations (Schoenly, 1992; Amendt *et al.,* 2004; Harvey *et al.,* 2016). Figures 1.1.a and 1.1.b highlight the increase in forensic entomology research worldwide. The European Association for Forensic Entomology (EAFE) was founded in 2002 and holds an annual meeting in different cities in Europe; the Association aims to bring together European forensic entomologists to unify the discipline, share research and create common protocols (Bourguignon, 2018). Similarly, the North American Forensic Entomology Association (NAFEA) was founded in 2005 (Byrd, 2018), and fulfils a similar function in that continent as the EAFE does in Europe.

Figure 1.1.a

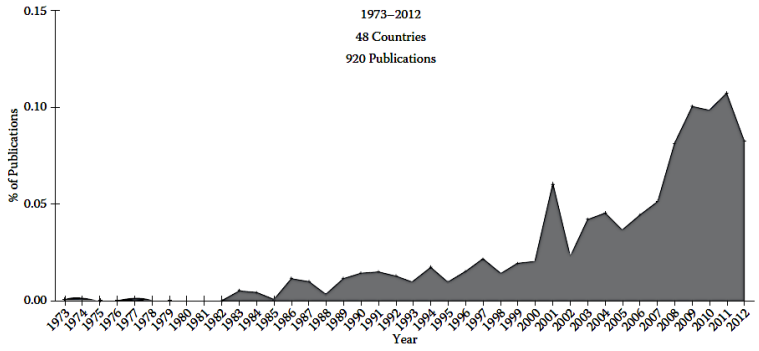

Figure 1.1.b

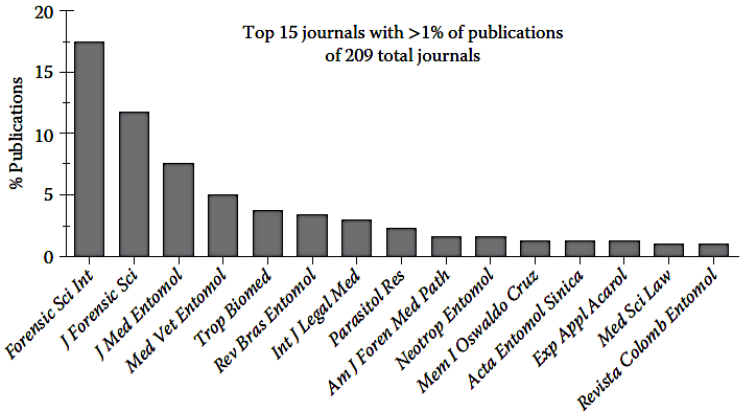

Figure 1.1.a: Summary of all publications that reference 'forensic entomology' from 1973 to 2012. **Figure 1.1.b:** Journals that published manuscripts that referenced 'forensic entomology' from 1973 to 2012. (Tomberlin and Benbow, 2015, pp 418 and 419).

1.3 An overview of forensic entomology

The minPMI interval can be estimated by forensic entomologists through the application of a range of different techniques, including: the identification of species of insect found on a corpse; determining their stage of development; examining the succession of species on the corpse and using the known rates of development combined with the consideration of the extrinsic factors acting on the corpse, most importantly temperature (Amendt *et al.,* 2004). There are two main

methods employed by forensic entomologists to determine minPMI. The first is by aging the insects associated with the body and the second is by studying the insect succession on the body (Harvey *et al.,* 2016). The first method generally provides the most accurate estimation of minPMI and is therefore used when possible, whereas the second method is utilised when the body has been deceased for a longer period of time and the aging of insect specimens on the body will no longer provide an accurate minPMI because the insects present are no longer the first colonisers (Mai and Amendt, 2012; Picard *et al.,* 2013; Harvey *et al.,* 2016). In order for minPMI to be accurate it is essential to age the insects that not only arrived first, but that consistently arrive first during the vast majority of decompositions. Blow flies are universally acknowledged as the primary colonisers of a body as they are olfactorily attracted to freshly decomposing remains and can arrive within hours of death (Wall *et al.,* 1992; Amendt *et al.,* 2011; Harvey *et al.,* 2016). After arrival the female blow flies proceed to lay their eggs on the remains and thus their colonisation of the body begins.

During the decomposition of a corpse there is a succession of colonisation by different species of insects and other invertebrates, such as mites (Amendt *et al.,* 2004). The entomologist and veterinarian, Jean Pierre Mégnin, was the first to recognise and describe the process of insect succession of a corpse in 1894 (Mégnin, 1894). During the decomposition process the physical and chemical changes that occur within the corpse represent different ecological niches that are best suited to different groups of invertebrates. The process of decomposition does not comprise discrete stages, but rather is a continuous and variable process, which can be manifested differently in every case. This is because of the many factors that affect decomposition, for example: temperature, humidity, precipitation, clothing, and the position of the body. However, for practical purposes the process of decomposition is often broken down into between three and eight major phases, for example: 'fresh', 'bloated', 'decay' and 'dry' (Figure 1.2) (Byrd and Castner, 2009). Up to 400 species of insects have been found on one pig carcass, coexisting, out competing and succeeding each other, during the entire decomposition process (Amendt *et al.* 2004). The most important orders of insects that are actively involved in the decomposition process (i.e. feed on the body) are: Diptera and Coleoptera (Smith, 1986; Byrd and Castner, 2009). A summary of the families of Diptera and Coleoptera is presented in Figure 1.2. Although not generally actively feeding on the body, Hymenoptera is also an important order of insect, as some species are parasitoids of the Dipteran species present on a decomposing organism (Smith, 1986; Frederickx *et al.*, 2013). Families of Hymenopteran parasitoids of necrophagous Diptera include: Braconidae, Pteromalidae, Encyrtidae, Diapriidae and Figitidae (Frederickx *et al.*, 2013)

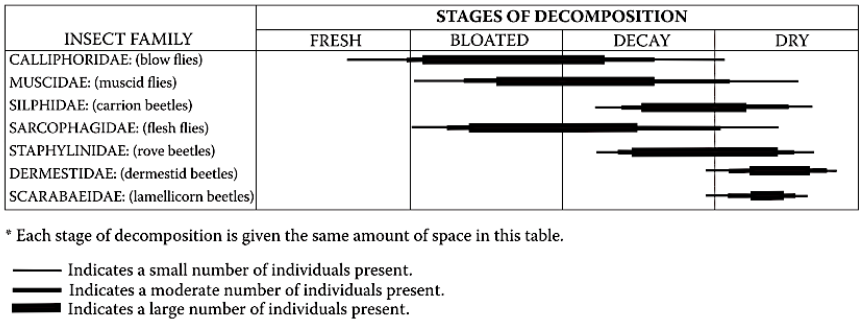

Figure 1.2: Families of insect most likely to be colonising a body during each stage of decomposition (from Byrd and Castner 2009, pp. 11).

In reality the decomposition process is continuous with no discrete stages (Matuszewski *et al.*, 2010)and therefore the difference in taphonomists' opinions as to how to divide and describe the decomposition process is understandable. Mégnin (1984) described eight stages of decomposition, associated with eight invasion waves of arthropods, but the process of decomposition has since been simplified to fewer stages. As previously noted, Byrd and Castner (2009) divided the decomposition process into four distinct stages; 'fresh', 'bloated', 'decay' and 'dry' (Figure 1.2). Benecke (2005) also divided decomposition into four stages but did not include 'fresh' as a separate stage, i.e. 'bloated', 'decay', 'fermentation', and 'mummification' or 'skeletonisation'. Campobasso *et al.* (2001) also separated the decomposition process into four stages: 'discolouration', 'bloating', 'liquefaction' and 'advanced decay' or 'skeletonisation'. Payne (1965) split the decomposition process into five stages: 'fresh', 'bloated', 'active decay', 'advanced decay' and 'dry'. This study will use Byrd and Castner's (2009) description of decomposition because it is the most cited, with the process divided into the fresh, bloated, decay and dry stages (Archer and Elgar, 2003; Amendt *et al.*, 2011; Benbow *et al.*, 2013).

Blow flies are associated with the decomposition of a corpse from the fresh to decay stages of decomposition – they are generally the primary colonisers of a corpse and are therefore acknowledged as the most important insects in determining minPMI (Smith, 1986; Hall, 2007; Amendt *et al.,* 2011). To determine minPMI most accurately, it is necessary to study the insect specimens that colonise the body most rapidly after death, thus by aging the oldest of these insects minPMI can be determined.

1.4 Blow fly lifecycle

Blow flies, like all insects, are poikilothermic. They are unable thermally to regulate their body temperature to maintain a constant temperature and rather their body temperature fluctuates with their environment. As such the rate of development of blow flies is temperature dependent, whereby they develop faster in warmer weather and slower in colder weather (Johnson *et al.,* 2014). There are species specific threshold temperatures below and above which development will not occur (Sharpe and DeMichele, 1977; Jarosík *et al.,* 2002). The specific temperature requirements for different blow fly species vary greatly as does their ability to withstand temperature extremes. This is highlighted by the different lower development thresholds exhibited between species: *Lucilia sericata* has a lower development threshold of 11 °C (Greenberg, 1991; Pitts and Wall, 2004), *Protophormia terraenovae* (Robineau-Desvoidy) has a minimum development threshold of ~ 9 °C (Grassberger and Reiter, 2002), the lower development threshold for *Cochliomyia macellaria* is 10 - 12.5 °C (Greenberg, 1991) and the lower development threshold for *P. regina* is 4.2 °C (Greenberg, 1991). *Calliphora vicina* has one of the lowest development thresholds of 2.5 - 4 °C (Greenberg, 1991).

While some blow fly species are larviparous and lay live larvae, for example *Calliphora pattoni* (Aubertin), most are oviparous (Nandi, 2002). Oviparous species lay fertilised eggs onto a substrate suitable for their offspring, mainly carrion (Amendt *et al.* 2011). Eggs are laid in batches and each batch usually contains a few hundred eggs, but the number of eggs per batch varies between species. For example *Lucilia eximia* (Wiedemann) lays eggs in batches of 30 to 70 (Greenberg and Szyska, 1984), *Compsomyiops boliviano* (Mello) lays between 75 and 250 eggs per batch (Greenberg and Szyska, 1984), *Hemilucilia hermanlenti* (Mello) lays between 100 and 150 eggs (Greenberg and Szyska, 1984), while *Lucilia sericata* (Meigen), *C. vicina* (Robineau-Desvoidy) and *C. vomitoria* (Linnaeus) all lay about 200 eggs per egg batch (Greenberg and Szyska, 1984; Davies, 2006) and *L. cuprina* (Wiedemann) lays eggs in batches of around 450 (Zied *et al.,* 2003).

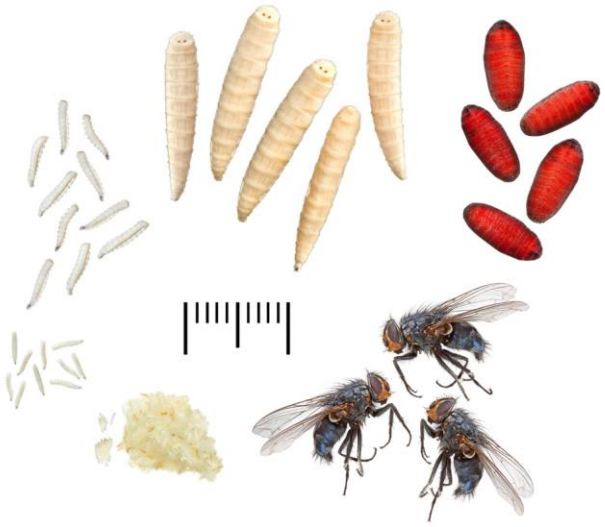

Figure 1.3: The general lifecycle of blow fly species. Moving clockwise from bottom right: adults lay eggs that hatch into 1st instar larvae, which moult to 2nd then 3rd instar larvae. The final stage shows the puparia prior to adult emergence. (© The Natural History Museum, London). Scale shows 1 cm.

Blow flies are holometabolous, meaning that they undergo complete metamorphosis. Once the eggs have hatched into 1st instar (second developmental stage) larvae, the latter feed on the substrate (e.g. dead body) and continue to do so through to the end of the 3rd instar (Figure 1.3). Once the larvae have finished feeding, they generally disperse from the substrate to find a suitable pupariation site. The larval cuticle of the post-feeding larvae constricts, darkens and hardens, forming the puparium (Martín-Vega *et al.*, 2016, 2017). The pupa undergoes metamorphosis within the puparium and eventually emerges as an adult fly (Figure 1.3). The lifecycle continues as the mature adult female fly mates and then lays eggs on a suitable substrate. Oviposition occurs during daytime only; Barnes *et al*. (2015) observed no nocturnal oviposition (by *C. vicina, C. vomitoria* or *Lucilia* species) during a 3 year study (2011-2013) in England. Examples of the time spent in each development stage by some blow flies at different temperatures are summarised in Table 1.1.

Table 1.1: Summary of the literature concerning the development time of different blow fly species at different temperatures.

Species	Temp.	Development stage						References
		Egg	1st instar	2nd instar	3rd instar	Post-feeding	Puparial	
Lucilia sericata	22 °C	23	27	22	22	108	143	Greenberg (1991)
L. sericata	29°C	18	16	16	22	94	130	Greenberg (1991)
L. sericata	25 °C	14	16	19	36	87	125	Grassberger and Reiter (2001)
Phormia regina	22 °C	20	25	25	25	125	117	Greenberg (1991)
P. regina	29 °C	18	12	15	25	110	99	Greenberg (1991)
Protophormia terraenovae	15 °C	69					384	Grassberger and Reiter (2002)
P. terraenovae	20 °C	31	NA	NA	NA	NA	216	Grassberger and Reiter (2002)
P. terraenovae	25 °C	19	NA	NA	NA	NA	144	Grassberger and Reiter (2002)
P. terraenovae	30 °C	11	NA	NA	NA	NA	120	Grassberger and Reiter (2002)
P. terraenovae	35 °C	9	NA	NA	NA	NA	96	Grassberger and Reiter (2002)
Calliphora vicina	10 °C	88	NA	224	NA	355	980	Greenberg (1991)
C. vicina	12.5 °C	38	49	58	65	199	660	Greenberg (1991)
C. vicina	19 °C	19	22	23	65	118	336	Greenberg (1991)
C. vicina	25 °C	14	18	19	26	122	261	Greenberg (1991)
C. vomitoria	12.5 °C	65	55	60	278	156	718	Greenberg and Tantawi (1993)
C. vomitoria	23 °C	22	25	19	128	86	247	Greenberg and Tantawi (1993)
C. vomitoria	29 °C	17	11	18	88	74	NA	Greenberg and Tantawi (1993)
C. vomitoria	35 °C	12	18	12	44	NA	NA	Greenberg and Tantawi (1993)

In order to accurately estimate the minPMI, it is important to identify the oldest specimens associated with the decomposition of the body. Due to the high variability of its duration (both within and between species), the post-feeding larval dispersal stage is key to estimating the minPMI (Kočárek, 2001; Gomes and von Zuben, 2005; Gomes *et al.,* 2006a; Arnott and Turner, 2008).

1.5 Ecology of dispersal

This thesis examines the post-feeding larval stage of the calliphorid lifecycle. As outlined in Section 1.4 this developmental stage is often characterised by dispersal from the food source. The dispersal discussed in this study is the active dispersal of organisms, which describes their movement from one location to another. While the puparia of some calliphorid species, such as *P. terraenovae*, can often be found on the body, most species disperse away from the body to pupariate and this phenomenon is well documented in the literature (Tessmer and Meek, 1996; Andrade *et al.*, 2002; Gomes and von Zuben, 2005; Arnott and Turner, 2008; Lewis and Benbow, 2011).

The dispersal of populations of a species is also well documented throughout the animal kingdom: migratory birds (Kokko, 1999), fish (Gross *et al.,* 1988), mammals (Avgar *et al.,* 2014), zooplankton (Zaret and Suffern, 1976) and insects (Drake and Gatehouse, 1995). Reasons for these animal migrations include: increase in availability of food resources, an increased habitat range, increased reproductive success and the evasion of predators (Zaret and Suffern, 1976; Gross *et al.*, 1988; Drake and Gatehouse, 1995; Kokko, 1999; Avgar *et al.,*2014). The factor that seems most applicable to the post-feeding larval dispersal of blow flies is the evasion of predators, because dispersal diminishes the chances of predation from opportunistic predators such as other insects and vertebrate scavengers, as these predators are also attracted to the body (Gomes *et al.,* 2006a). Although there could be a number of reasons why larval dispersal occurs: for example, the decomposition of the body creates a toxic environment and the larvae themselves excrete ammonia, exacerbating this (Kočárek, 2001). In fact, the decomposition of a body during the bloat stage can destroy the underlying natural fauna (Parmenter and MacMahon, 2009; Tomberlin *et al.*, 2011), potentially up to 14 cm deep and moreover, complete floral and faunal reinvasion of the soil may not occur until after one year of the body's initial emplacement (Bornemissza, 1957).

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1.5.1 Horizontal and vertical dispersal of post-feeding larvae

1.5.1.1 Horizontal dispersal of post-feeding larvae

It is essential to know the distance that post-feeding larvae may move from their food source at a crime scene, as it is necessary to decide how far from the body to search for post-feeding larvae, puparia and/or empty puparial cases.

Some studies have shown that certain species of larvae may travel up to 50 m (150 ft) from their origin (species not specified) (Byrd and Castner, 2009), while other studies have noted that some species do not move from the body at all and puparia can be found under the body and in the crevices of clothes. Amendt *et al.* (2007), do not specify species, but Pohjoismäki *et al.* (2010) note that this phenomenon applies to *Protophormia terraenovae* and *Lucilia sericata*. The distance larvae may disperse from their food source is species specific and this is most likely due to the different pupariation environments required by different species (Godoy *et al.,* 1995). While this study focuses on forensically important UK blow flies, it is important to highlight that discrepancies are common in the literature concerning many different blow fly species worldwide. Table 1.2 is a summary of the literature reporting distances dispersed by different blow fly species.

Species	Distance dispersed from the food source	Substrate	Reference
Not specified	On food source	Not specified	(Amendt *et al.*, 2007)
Not specified	50 m	Not specified	(Byrd and Castner, 2009)
Calliphora vicina	3 – 8.1 m	Sawdust	(Greenberg, 1990)
Chrysomya albiceps	< 1 m	Sawdust	(Andrade *et al.*, 2002)
C. albiceps	< 1 m	Soil	(Tantawi *et al.*, 1996)
C. albiceps	> 1 m	Sawdust	(Gomes and von Zuben, 2005)
Chrysomya megacephala	< 1 m	Sawdust	(Andrade *et al.*, 2002)
C.megacephala	< 1 m	Sawdust	(Godoy *et al.*, 1995)
C.megacephala	> 1 m	Sawdust	(Gomes *et al.*, 2005)
Chrysomya rufifacies	On food source	Not specified	(Byrd and Castner, 2009)
C. rufifacies	< 3.3 m	Sawdust	(Greenberg, 1990)
C. rufifacies	On food source	Soil	(Richards and Goff, 1997)
Cochliomyia hominivorax	0.6 – 6 m	Soil	(Travis *et al.*, 1940)
Cochliomyia macellaria	< 1 m	Sawdust	(Andrade *et al.* 2002)
C. macellaria	< 1 m	Sawdust	(Godoy *et al.*, 1996)
C. macellaria	< 1 m (the majority of puparia recovered during the study)	Sawdust	(Greenberg, 1990)
C. macellaria	5.1 m (< 0.5 % of the puparia recovered during the study)	Sawdust	(Greenberg, 1990)
C. macellaria	< 1 m	Soil	(Tessmer and Meek, 1996)
Lucilia illustris	3 m	Soil	(Nuorteva, 1970)
Lucilia caesar	3 m	Soil	(Nuorteva, 1970)
Lucilia sericata	6.4 m	Soil	(Cragg, 1955)
L. sericata	3 – 8.1 m	Sawdust	(Greenberg, 1990)
L. sericata	On food source	Clothes	(Pohjoismäki *et al.*, 2010)
L. sericata	4.6 m	Soil	(Tessmer and Meek, 1996)
Phormia regina	On food source	Sawdust	(Greenberg, 1990)
P. regina	6 m	Soil	(Lewis and Benbow, 2011)
P. regina	26 m *	Soil	(Lewis and Benbow, 2011)
P.regina	> 4.6 m	Soil	(Tessmer and Meek, 1996)
Protophormia terraenovae	On food source	Clothes	(Erzinçlioğlu, 1996)
P. terraenovae	On food source	Clothes	(Pohjoismäki *et al.*, 2010)

Table 1.2 highlights the discrepancies reported in the literature concerning the distances dispersed by post-feeding larvae of different species of blow fly. With regards to *C. macellaria*

most of the literature agrees that the species tends to disperse less than 1 m from the food source. Greenberg (1990) reported that the majority of their study population dispersed less than 1 metre, with less than 0.5 % dispersing up to 5.1 m. Within populations there will be differences seen between different individuals as is natural and therefore the behaviour of the majority is far more important. Greenberg (1990) reported recovering an even distribution of *L. sericata* puparia throughout their experimental apparatus, up to 8.1 m. The even distribution shows that this species is capable of dispersing up to 8.1 m under certain circumstances. The study conducted by Lewis and Benbow (2011) with *P. regina* consisted of six replicate experiments, during one of which the soil was waterlogged due to heavy rainfall and in only this replicate, the larvae dispersed up to 26 m (during the other replicates *P. regina* was recovered up to 6 m from the food source [Table 1.2]). Lewis and Benbow (2011) concluded that the extended dispersal seen in one of the replicates was due to the larvae's inability to penetrate the waterlogged soil for subsequent pupariation (Section 1.5.1.3). Examination of the literature highlights the variability of post-feeding horizontal larval dispersal distances and the author concludes that this stage of development is sensitive to different environments, e.g. different substrates and moisture content.

Not only does the post-feeding dispersal distance vary between species, but differences have been found between different populations of the same species (Greenberg, 1990). One study even showed great variances in the pre-pupariation movement of *C. macellaria* from the same population during consecutive runs of the same experiment, i.e. when *C. macellaria* individuals from the same population were exposed to the same conditions, roughly half of them pupariated on the food source, while half travelled up to 2.4 m from the food source to pupariate (very few were recovered at intermediate distances) (Greenberg, 1990). No explanation was given for this difference, other than the variability of nature. This study highlights the difficulties in estimating the distances that larvae will travel and emphasises the need for a more thorough study that considers the many factors at play.

1.5.1.2 Vertical dispersal of post-feeding larvae

Where possible, dispersing larvae burrow into their environment once they have found a suitable pupariation site (Gomes *et al.*, 2006a) and, as with distance, burial depth can be species specific (Gomes *et al.*, 2005). This may be due to competition for pupariation sites, especially in predatory species such as *C. albiceps*, whose larvae are facultative predators of other Calliphorid larvae (Faria *et al.*, 1999). *Chrysomya megacephala* has been shown to burrow deeper when in the presence of *C. albiceps*, and this may be a strategy to avoid predation (Gomes *et al.*, 2005).

Although newly emerged adult blow flies require more energy to resurface when, as larvae, they burrowed deeper, it is not necessarily detrimental to the individual. In fact *C. macellaria* and *P. terraenovae* adults can, when forced to, successfully surface after being buried up to 50 cm deep in soil, moreover 40% of *C. macellaria* buried 120 cm deep successfully emerged (Balme *et al.,* 2012). Gunn and Bird (2011) reported that when post-feeding larvae of *C. vomitoria* and *L. sericata* were placed on the surface they burrowed immediately into the soil to pupariate and some were recovered from up to 25 cm deep, although the majority of *L. sericata* pupariated in the top 2 cm of soil and the majority of *C. vomitoria* pupariated in the top 8 cm of the soil. Moreover, all of the adult flies that emerged (even from 25 cm deep), successfully surfaced from the soil (Gunn and Bird, 2011). Most species, however, can usually be found naturally within the top 5 cm of the dispersal substrate (Godoy *et al.,* 1995).

There are many environmental factors that affect burial depth. Species such as *C. megacephala* show an increased burial depth when exposed to longer periods of light (Gomes *et al.,* 2006b): there was a statistically significant increase in burrowing depth due to increased photoperiod, L:D 0:24 mean = 3.1 cm depth, L:D 12:12 mean = 4.3 cm, L:D 24:0 mean= 8 cm. Certain species burrow deeper (*Lucilia cuprina*) while others burrow less deep (*C. albiceps*) when exposed to extreme high and low temperatures (Gomes *et al*., 2009). Moreover, for some species, such as *C. albiceps*, deeper burial has been associated with greater dispersal distances (when compared to the average depth burrowed when this species dispersed less horizontally) (Gomes and von Zuben, 2005).

Substrate compaction greatly affects burial depth and there is a strong negative correlation between high soil compaction and puparial depth (Cammack *et al.,* 2010; Frederickx *et al.,* 2014). The mean burial depth for *L. sericata* in uncompacted soil was shown to be 4.4 cm, compared to highly compacted soil, where the mean burial depth was 0.5 cm (Cammack *et al.,* 2010). This negative relationship may be due to the reduced availability of pore space in the substrate, and thus the available oxygen in more compacted substrates (Ullyett, 1950; Geden, 2002; Cammack *et al.,* 2010), or it may simply be due to the increased difficulty of burrowing into compacted soil. Conversely, increased burial substrate compaction has been shown to reduce the level of parasitism by parasitoids of calliphorids which may be due to the reduction in available oxygen in the soil in highly compacted substrate (Frederickx *et* al., 2014), or due to the inability of parasitoids to burrow into compacted soil. It has also been shown that pupariating close to the surface of the substrate may increase the calliphorid host's susceptibility to parasitoids and other predators, as although most parasitoids cannot penetrate the burial substrate at all, some are able to penetrate up to 5 cm deep (Guillén *et al.,* 2002).

The many factors that appear to influence burial depth of post-feeding larvae underline the need for this area to be more comprehensively studied, specifically investigating the multiple interactions between the different factors.

1.5.1.3 The effect of the dispersal substrate on post-feeding larval dispersal

Substrate may have a significant effect on the speed and distance of larval dispersal, but the topic has been under studied. While some studies mention the dispersal substrate as a cause for certain dispersal behaviour (Arnott and Turner, 2008; Robinson *et al.*, 2018), most studies (Tessmer and Meek, 1996; Gomes *et al.*, 2005; Charabidze *et al.*, 2008) ignore this factor and one even stated that saturation of the substrate has no effect on the dispersing larvae (Miller, 1929). Miller (1929) used silk ribbon in his experiments (such that larvae were only able to disperse along the silk ribbon) and concluded that the saturation of ribbon, and therefore any dispersal substrate, has no effect on the dispersal speed and therefore the type of dispersal substrate can be discounted. Arnott and Turner (2008) highlighted the need for a more comprehensive investigation into the effect of the dispersal substrate on the time spent dispersing by post-feeding larvae; their preliminary work, which involved using a smooth plastic surface, suggested that dispersal time is indeed dependent on the dispersal substrate (Arnott and Turner, 2008).

The composition of the dispersal substrate will greatly influence the distance and speed that larvae travel. Robinson *et al.* (2018) showed that unfavourable pupariation mediums, such as plastic flooring, result in further post-feeding dispersal distances in comparison to suitable pupariation mediums, such as sawdust or carpet, which may result in very short dispersal distances (experiments conducted using *L. sericata*). Speeds of up to 30 metres/hour by *C. vicina* dispersing on a smooth, plastic indoor substrate have been observed (personal observations, unpublished data). The density of the substrate has been regarded as an important factor, with soil compaction and water retention associated with longer surface dispersal distances (Byrd and Castner, 2009; Lewis and Benbow, 2011). The dispersal substrate is rarely homogenous and therefore the speed and distance of the dispersing larvae will be highly variable (Cammack *et al.,* 2010). Moreover, larvae have been shown to move along the path of least resistance and even upslope when the soil closer to the corpse is waterlogged, showing an ability of the larvae to avoid an unfavourable dispersal substrate (Lewis and Benbow, 2011).

In a natural setting, post-feeding larvae may encounter an even more adverse setting than a substrate with a high level of heterogeneity, for example, a waterlogged substrate or a substrate with obstacles such as plant roots. Lewis and Benbow (2011) recorded post-feeding larval dispersal of up to 26 m from the feeding substrate; in this case the dispersal substrate was extremely waterlogged and the larvae were forced to disperse until a suitable pupariation site was found, i.e. uphill where the ground was not waterlogged (Section 1.5.1.1). Moreover, Lewis and Benbow's (2011) study highlights the inaccuracy of the statement made by Miller (1929), that the saturation with water of the dispersal substrate has no effect on post-feeding larval dispersal (Miller, 1929).

The dispersal substrate is a very important factor affecting the dispersing larvae, not only affecting the speed and distance travelled, but also affecting the burial depth of the dispersing larvae (Gomes *et al.,* 2003; Cammack *et al.,* 2010). It is also important to note that the composition of the dispersal substrate is important as it has been suggested that a dispersal substrate that can 'clump', creating free space allows larval movement; conversely a substrate that does not 'clump' impedes larval movement (Dimou *et al.,* 2003). These 'globular aggregates' may help larval movement through the substrate and allow deeper penetration (Dimou *et al.,* 2003).

Arnott and Turner (2008) reiterated the need for further studies that examine the effect of terrain on dispersing larvae of the "main species" (the most common species of forensic importance) in order to provide more accurate estimations of minPMI. The effect of the dispersal substrate on dispersing larvae is clearly an area that needs to be more thoroughly examined.

1.5.1.4 Active aggregation of post-feeding larvae prior to pupariation

Aggregation describes the active accumulation of larvae during different stages of development. Aggregation during the active feeding larval stages, prior to dispersal, is well documented (Slone and Gruner 2007; Rivers *et al.,* 2011). Calliphorid larvae feed gregariously conferring benefits such as: larval cooperation (exodigestion), the acceleration of larval development and an overall increase in larval survival (Charabidze *et al.,* 2011; Boulay *et al.,* 2016). Aggregation prior to pupariation, during the dispersal stage of development, is not well studied. Some species aggregate during their dispersal and different species have been shown to aggregate more than others (Godoy *et al.,* 1996). One study by Lewis and Benbow (2011) observed novel mass aggregation patterns of *P. regina* larvae leaving a corpse and travelling up to 26 metres before individually dispersing in a 360° radius and burrowing (Lewis and Benbow, 2011). Aggregation

may aid in dispersal speed and in reducing interspecific predation, by creating a "safety in numbers" effect (Reigada and Godoy, 2005; Lewis and Benbow, 2011). The interspecific competition of blow fly larvae is well documented, for example the predation by facultative predator calliphorid larvae on other calliphorid larvae and the interspecific larval competition for resources, such as food (Andrade, *et al.* 2002; Reigada and Godoy, 2005; Rosa *et al.,* 2006). *Cochliomyia macellaria* has been shown to favour aggregation and *Chrysomya putoria* (Wiedemann) and *C. megacephala* have also been shown to aggregate, but to a lesser extent (Godoy *et al.,* 1996). Conversely it has been demonstrated that *C. megacephala* only aggregates when there are predatory species of blow fly present (Reigada and Godoy, 2005).

During choice experiments, *L. sericata* and *C. vomitoria* larvae showed a preference for aggregative feeding (Boulay *et al.,* 2016). The reasons behind post-feeding larval aggregation are generally under studied. Some studies have offered no explanation for the aggregation of pre-puparia dispersing larvae, but Lima *et al.* (2009) suggest that the neighbourhood interactions of dispersing larvae and their perceived ability to detect other larvae in the pupariation substrate may be involved and these neighbourhood interactions between larvae predisposes them to aggregate. Fouche *et al.* (2018) showed that both *C. vomitoria* and *L. sericata* post-feeding larvae exhibited a preference for actively aggregating in areas previously marked by other larvae of both species; *L. sericata* showed a preference for areas marked by other *L. sericata* larvae and both species preferentially selected areas marked by greater numbers of larvae. Their study highlights potential for calliphorid larvae to actively aggregate in the post-feeding larval stage and therefore produce aggregations of puparia in the dispersal substrate. It has also been demonstrated that pre-puparial larvae in the absence of a suitable dispersal substrate will aggregate together (Holmes *et al.*, 2013). It has been suggested that this behaviour is a thigmotactic response to the surrounding puparia and pre-puparial larvae (Holmes *et al.,* 2013). This theory is supported by the dispersing larva's ability to detect others in the pupariation substrate (Lima *et al.*, 2009). Some studies have suggested the opposite, i.e. that the larva's ability to detect neighbouring puparia and larvae in the pupariation substrate encourages them to move further away to a less crowded section of substrate (Gomes and Zuben, 2005).

Dispersing *C. vicina* larvae may exhibit aggregation behaviour by following trails left by other larvae of their species (Arnott and Turner, 2008). The aggregation of some blow fly species, such as *L. sericata*, has been observed to be active and ultimately associated with the chemical cues given by other larvae. In a study by Boulay *et al.* (2013) aggregation took place quickly and was reinforced with time, contact and/or odour-mediated signals involved in aggregation behaviour (Boulay *et al.,* 2013). It has also been hypothesised that local communication occurs between

blow fly larvae due to ground marking signals that are passively disseminated by dispersing larvae (Boulay *et al.*, 2016). These signals have been shown to have an intraspecific attractive/retentive affect (Boulay *et al.,* 2013). The chemical exact composition of this signal has not yet been identified, but its presence has been confirmed (Boulay *et al.,* 2015). Preliminary gas chromatography evidence suggests that the signal is a cholesterol derivative compound and that it could be produced and recognised by multiple blow fly species (Boulay *et al.,* 2016).

A high density of prey attracts greater numbers of predators and parasitoids (Hassell, 1978; Taylor, 1984). It has been suggested that as parasitoids are attracted to puparia by visual and chemical cues, puparial aggregation should increase the chance of parasitism (Cammack *et al.,* 2010). However puparial aggregation has been seen to increase in the presence of parasitoids and therefore aggregation may confer a "safety in numbers" strategy (Cammack *et al.,* 2010). This study will aim to determine which species aggregate during the pre-puparial stage and under what conditions aggregation is enhanced or reduced.

1.5.2 The direction of larval dispersal

Another consideration of post-feeding larval dispersal about which forensic entomologists are unable to agree is the direction from the food source in which dispersing larvae may travel. This would clearly not be pertinent to crime scene applications of forensic entomology if the larvae do not disperse from the body. However, when some larvae may move up to 26 m from the body, the need to concentrate collection efforts in a specific direction is emphasised (Lewis and Benbow, 2011).

Some studies have suggested that certain species of blow fly move in preferential directions. In Louisiana, USA, blow fly larvae have been found to disperse in a SE direction in summer and spring and in a SW direction in autumn, apparently due to an 'innate directional preference' rather than other environmental factors (Tessmer and Meek, 1996). Another study found that, during six replicated experiments, larvae always moved in a N-NE direction (Lewis and Benbow, 2011). In comparison to the findings of Tessmer and Meek (1996), Lewis and Benbow (2011) described *P. regina* as always traveling upslope and predominantly in a N-NE direction and again this appears to be due only to an innate directional preference exhibited by the larvae. *Protophormia terraenovae, C. macellaria, C. albiceps, C. putoria* and *C. megacephala* have all been described as species that show no preferential directionality while dispersing, despite the fact that *P. terraenovae* moves very little distance from the food source (Roux *et al.* 2006; Arnott and Turner,

2008; Lima *et al.,* 2009). The idea of dispersing calliphorid larvae having an 'innate directional preference' (i.e. a preferred direction, regardless of the environmental conditions) seems unlikely. Moreover each study that has stated that certain species of larvae possess an 'innate directional preference' (Tessmer and Meek, 1996; Lewis and Benbow, 2011) has made this conclusion based on the results of one study conducted in the same area. It seems much more likely that the 'innate directional preference' exhibited by these dispersing larvae was due to the specific circumstances of the study area, i.e. the direction of sunlight at that time of year (larvae move away from light [Section 1.5. 6]) or the compaction of the soil (larval penetration is not possible in highly compacted soil and therefore larvae will attempt to disperse until penetration is possible [Section 1.5.1.3]). The direction of larval dispersal is most likely situation specific rather than species specific.

Of importance is the lack of homogeneity in the natural environment, therefore larval dispersal will be affected as they encounter obstacles in their path, such as stones and logs (Section 1.5.1.3; Cammack *et al.,* 2010). As previously stated (Section 1.5.1.3), waterlogged soil is not an ideal pupariation environment and therefore, dispersing larvae in this situation will disperse upslope if possible. Soil in a natural outdoor environment tends to be less homogenous than that used in the laboratory, so larvae may not pupariate as deeply. When an object is encountered, larvae change direction, and larvae in the field may therefore burrow laterally more often than in the laboratory. It is also important to note that dispersal has been shown to be aggregative and therefore, while the first dispersing larva's direction may be random, the subsequent larvae will tend to follow the trail of the first to disperse (Arnott and Turner, 2008).

1.5.3 Puparial weight

Although there appear to be benefits to blow fly species that leave the food source to pupariate, including a reduction in the risk of predation (Gomes *et al.,* 2006a), it has been noted that dispersing greater distances may have a negative impact on the reproductive fitness of the individual (Godoy *et al.,* 1995; Gomes *et al.* , 2005; Mai and Amendt, 2012).

Some studies have used puparial weight as a measure of fitness, whereby reduced weight signifies reduced fitness (Godoy *et al.,* 1995; Gomes *et al.* , 2005; Mai and Amendt, 2012). The fitness of an individual refers to the health of the individual and therefore its reproductive success. A lower puparial weight has been associated with greater distances travelled by the larvae and, therefore, it was concluded that travelling further from the corpse may have a

negative impact on the health of the adult fly (Gomes and von Zuben, 2005). This study however, did not note the age of the puparia as the pupariation time was unknown (Gomes and von Zuben, 2005). Indeed, the puparial weight of *C. macellaria* and *C. albiceps*- the species used in the study- decreased as the distances the larvae dispersed increased (Godoy *et al.* 1995; Gomes *et al.* 2006b). However, the pupariation time and hence the age of the puparia was unknown for each study (Godoy *et al.* 1995; Gomes *et al.* 2006b).

Zajac and Amendt (2012) showed that puparial weight was not an accurate measurement of the age or fitness of the blow fly specimen and therefore should not be used to estimate either. Puparial weight has been shown to undergo changes during the entire puparial period and changes can occur within hours, due to dramatic water loss, especially directly after pupariation (Zajac and Amendt, 2012).

Fitness is more accurately represented by the size of the adult fly as larger female flies produce more eggs and are thus reproductively fitter (Mackerras, 1965). Measuring the dm-cu cross vein is an appropriate way to represent the size of an adult fly (Ireland and Turner, 2006; Mai and Amendt, 2012). It has been shown that an increase in the time spent dispersing in the larval stage results in smaller adult blow flies, indicated by the measurement of the d-cu cross vein of the wing for both *C. vicina* and *L. sericata* (Arnott and Turner, 2008; Mai and Amendt, 2012). Moreover, a dispersal time of over 48 hours, when compared with 12 hours, has been shown to result in significantly smaller *L. sericata* adult flies (Mai and Amendt, 2012). Arnott and Turner (2008) noted a significant but small difference in the resultant adult size from larvae that had spent longer dispersing. It has been suggested that this decrease in adult size, with increasing distances travelled, may be because more energy is expended the further the larva travels (Arnott and Turner, 2008).

In order for larvae to find their optimum pupariation site they may have to travel further. This appears to have a negative impact on the size and therefore fitness of the adult fly. There must therefore be a trade-off between the optimum pupariation site and the health of the adult individual.

1.5.4 Intra-puparial duration

The length of time spent in the puparial stage (intra-puparial period) is essential to calculating minPMI. It is generally assumed that puparial period is fixed, dependent on species and

temperature only (Greenberg and Szyska, 1984; Amendt *et al.,* 2011). Almost all studies conducted to generate datasets for minPMI calculations do not consider the variability of the intra-puparial period. The studies were conducted under laboratory conditions and the factors that may affect intra-puparial period (such as time spent dispersing) were not considered (Greenberg and Szyska, 1984; Amendt *et al.,* 2011). In nature the duration of the intra-puparial duration may be highly variable, as there are more factors at play than just temperature and species (Arnott and Turner, 2008; Cammack *et al.*, 2010; Mai and Amendt, 2012). Thus, understanding the factors that affect this period are essential so that they can be incorporated into minPMI estimations, therefore reducing inaccuracies.

Studies have shown that the overall developmental time from egg to adult varies because the post-feeding larvae may spend different times in the dispersal stage. In fact, prolonged larval dispersal increases puparial period (Mai and Amendt, 2012), sometimes by as much as twice the length of the dispersal time (Arnott and Turner, 2008). The extent that the puparial period is affected by longer dispersal times is species specific. For example *L. sericata* dispersal times of over 24 hours increases the puparial period (6.9 hour increase in total development time for 24 hours of dispersal and a 29 hour increase in development time when 48 hours were spent dispersing) (Mai and Amendt, 2012), while the puparial period of *C. vicina* increases significantly when dispersal times exceed only five hours (Arnott and Turner, 2008). Moreover, Arnott and Turner (2008) suggest that when increased dispersal times are not considered, minPMI estimations will be underestimations of the actual PMI. The puparial period of *C. vicina* that disperse for six hours will be 1.25 days longer than the currrent minPMI estimations would suggest; eight hours dispersing would be 1.5 days longer and 24 hours spent dispersing would be 2.1 days longer (Arnott and Turner, 2008).

As previously discussed, soil compaction affects the dispersal rate and the burial depth of larvae, and soil compaction also affects the intra-puparial period. *Lucilia sericata* in low compacted soil developed faster than in highly compacted soil and Cammack *et al.* (2010) suggested that this may be due to the increased energy expenditure needed for the penetration of highly compacted soil, resulting in less energy available for pupariation and therefore more time spent in the pupariation period. The puparial period was between 10.5 and 18.8 hours longer (at 25°C) when compared to the puparial period of puparia in uncompacted soil (Cammack *et al.,* 2010). However, the exact time of pupariation of each specimen was unknown and therefore the conclusions may be speculation

The puparial period of *L. sericata* has been shown to be shorter when the puparia are in the presence of parasitoids and increased aggregation due to presence of parasitoids (Cammack *et al.,* 2010). This strategy employed by the species to avoid parasitism shows the variability of the puparial period and the apparent ability of the individual to control it. There are many factors that affect the puparial period and these factors and their inter-relationships are not easily explained.

1.5.5 Environmental effects on dispersal of post-feeding larvae

Given that blow flies are poikilothermic, temperature is commonly stated as the most important factor affecting the rate of development (Godoy *et al.,* 1996; Gomes *et al.,* 2006a; Arnott and Turner, 2008; Holmes *et al.,* 2013). Temperature not only affects the rate of development of blow flies, but has also been shown to affect the speed of larval dispersal (Charabidze *et al.,* 2008). Some species, such as *P. terraenovae*, disperse faster horizontally when exposed to higher temperatures (Charabidze *et al.,* 2008). Charabidze *et al.* (2008) placed post-feeding *P. terraenovae* larvae on an 18.5 cm diameter petri dish in a thermostatic enclosure with ± 1 °C precision and video tracked the larval movements. In this way the changes in larval speed at six different temperatures (10, 15, 20, 23, 28 and 30 °C) were investigated (Charabidze *et al.,* 2008). The study determined that larval speed increased as a function of temperature and larval length and an allometric relationship was determined: speed (cm/min) = 5.45 x log [length(mm)] + 0.66 x temperature (°C) - 12.8 (Charabidze *et al.*, 2008). The increase in speed as a function of temperature may be because the higher temperatures increase the metabolic rate of the dispersing larvae (Gomes *et al.,* 2006a). Gomes *et al.* (2009) showed that the vertical dispersal of *C. albiceps* and *L. cuprina* was also affected by temperature, i.e. *L. cuprina* burrowed deeper when exposed to extreme high (30 °C) or low (15 °C) temperatures and *C. albiceps* burrowed less, when compared to their mean depths burrowed at 20 °C. Ambient temperature has also been shown to have a significant effect on the rate of colonisation of a body, by different blow fly species (Ody *et al.*, 2017). This is due to different oviposition temperature thresholds exhibited by each species; Ody *et al.* (2017) showed that *C. vicina* oviposition occurred between 10 and 35 °C, whereas *C. vomitoria* oviposition occurred between 16 and 40 °C and *L. sericata* oviposition occurred between 17.5 and 40 °C. Moreover, the temperature at which oviposition occurred was shown to affect the subsequent percentage survival of puparia for *C. vicina* and *C. vomitoria* but not for *L. sericata*. Survival to the puparial stage for *C. vicina* and *C. vomitoria* was highest when oviposition occurred at 25 °C and survival decreased significantly when oviposition occurred towards the upper and lower threshold temperatures. Whereas the puparial survival

rate of *L. sericata* was not significantly affected by the temperature at which oviposition occurred (Ody *et al.*, 2017). This study highlights that the effects of factors, such as temperature, on calliphorid development rates are species specific.

Larval weight is often used as a measure of the fitness of an individual and some studies have shown that temperature can have an effect on the weight and therefore the health of individuals. Puparial weight, however, does not seem to be a good measure of fitness [Section 1.5.4], and there is no study, to the author's knowledge, that shows that larval weight is not a good measure of fitness. Gomes *et al.,* (2009) showed that the weight of *L. cuprina* 3rd instar larvae decreased and *C. albiceps* adults increased in response to an increase in temperature; moreover the maximum larval weights recorded for each species were temperature specific. Developing blow fly larvae are not only exposed to the ambient temperature, but also to the heat generated when aggregated feeding occurs. During the late 2nd and throughout the 3rd instar, larval masses can produce heat that helps to maintain the optimum temperatures required for larval development (Campobasso *et al.,* 2001; Amendt *et al.,* 2011; Johnson *et al.,* 2014). Moreover, an increase of 35 °C has been recorded from the maggot mass in comparison with the ambient temperature (Anderson and VanLaerhoven, 1996).

Environmental factors are not the only factors that affect the pupariation behaviour of blow flies. A group of toxins produced by the larvae itself, called paralysins, are present throughout larval development (Chiou *et al.,* 1998). The concentration of these molecules increases in the larva and peaks just after pupariation, and the paralytic effect of these toxins may be responsible for the paralysis of the larvae prior to pupariation (Kotanen *et al.,* 2003; Mai and Amendt, 2012). Therefore, the pupariation process is driven not only by environmental factors but also by chemical cues.

1.5.6 The effects of photoperiod on post-feeding larvae and puparia

Photoperiod describes the period of light and dark experienced by an organism and is most often synonymous with day length (Beck, 1964). Blow fly larvae possess light receptors that enable them to detect light and they are negatively phototactic, moving away from sources of light (Hinnemann *et al.,* 2010). The larvae are capable of recognising different photoperiods, demonstrated by their ability to emerge from diapause when the photoperiod increases (Amendt *et al.,* 2004). The development rate of blow flies is affected by photoperiod, although photoperiod is often ignored in studies and this likely affects minPMI estimates (Amendt *et al.,*

2004; Harvey *et al.,* 2016). Photoperiod has a significant effect on the development of *P. regina*, *C. macellaria* and *C. vicina* and the overall development of these species was fastest when exposed to a cyclic photoperiod ([Light: Dark] 16:8 or 12:12 h) and slowest when exposed to constant light ([L:D] 24:0 h) (Fisher *et al.,* 2015).

As mentioned in Section 1.5.1.2, burial depth can be affected by exposure to light (photoperiod), for example *C. megacephala* burrow significantly deeper with increasing photoperiod: L:D 0:24 resulted in a mean vertical dispersal of 4 cm, L:D 12:12 resulted in a mean of 5 cm and L:D 24:0 resulted in a mean vertical dispersal of 6.5 cm (Gomes *et al.,* 2006b). Photoperiod also has an effect on diapause, which is induced in the offspring of adult female blow flies that are exposed to short photoperiods, as this is indicative of the short days of winter (Amendt *et al.,* 2004). Furthermore, it has been shown that the trigger that initiates dispersal from the food source may be light (Smith *et al.,* 1981). This latter factor supports studies that have shown certain species' dispersal to be diurnal (Schoenly, 1983; Arnott and Turner, 2008; Lewis and Benbow, 2011). However, larval dispersal has also been recorded at night and therefore this aspect of dispersal may also be situation specific (Green, 1954; Smith *et al.,* 1981; Kočárek, 2001).

It has been noted that photoperiod is an important factor that affects the rate of development of blow flies (Gomes *et al.,* 2006b). While this area is very under studied, as outlined in this Section it has an influence on many different features of the post-feeding dispersal of blow flies (Harvey *et al.,* 2016). dispersal.

1.6 Challenges of the current forensic practices, research and literature

A complete understanding of the post-feeding larval stage is essential for minPMI calculations. This stage is highly variable and even minor under or over estimates (of the age of the entomological evidence) can lead to serious miscalculations of minPMI estimates (Lewis and Benbow, 2011).

Of primary concern is the present practice for the crime scene collection of entomological material. Currently the recommendation for collecting entomological evidence from a crime scene is to search 360° of the area surrounding the body within a 10 m radius (Amendt *et al.,* 2007; Byrd and Castner, 2009). At outdoor scenes, it is recommended to take soil samples up to at least 2 m from the body and to a depth of 10 cm or more depending on the circumstances (Amendt *et al.*, 2007). The recommendation is to also take control samples of the soil, some

distance from the body, to obtain a background level of insect abundance and to ensure that all insects collected at the scene were from the body and not from other sources (Amendt *et al.*, 2007; Hall *et al.,* 2012). It is recommended to visually search the area around the body for other large mammals that could have contributed to the sampled insects (e.g. dead rabbits and foxes) (Amendt *et al.*, 2007; Hall *et al.,* 2012). At an indoor scene it is advised to check different rooms, not just the room the body was discovered in, for dispersing larvae or puparia (Amendt *et al.*, 2007). However, Lewis and Benbow (2011) suggest that when events such as an 'en masse larval dispersal' occur, there is a 33% chance that over 90% of the dispersing larvae would not be collected if the current forensic practice recommendations are followed. Lewis and Benbow's (2011) study highlights the need for a situation specific approach to understanding the entomological evidence at a crime scene. As dispersing and dispersed larvae are often the oldest specimens associated with the body and are therefore essential for the most accurate estimation of minPMI, overlooking specimens outside of the 10 m radius from the food source could result in significant underestimations of minPMI calculations.

In the UK, forensic entomologists are not usually engaged in the role full-time but act as consultants on a case-by-case basis and are considered expert witnesses under the criminal justice system (Hall *et al.,* 2015). Forensic entomologists may be required to attend crime scenes, but more often SOCOs (Scene of Crime Officers) or CSIs (Crime Scene Investigators) collect forensic evidence from a crime scene, including the entomological evidence. If, during the course of the case, it becomes essential to determine the minPMI, the evidence will be sent to a forensic entomologist for analysis. This process is referred to as forensic streamlining, whereby evidence is sent to experts for analysis as and when it is needed for the case (Hall *et al.,* 2015). In theory this process works; however, for the process to be effective there are assumptions made: the evidence is recognised and collected, the correct amount of evidence is recovered, the evidence is preserved correctly and the provenance of the evidence is accurately documented. Any errors in the collection process are especially relevant when collecting dispersing larvae and puparia. As these larvae may be found some distance from the body, they can be very easily overlooked. A quintessential example of this, is a case that was worked on by Dr Martin Hall, whereby he was sent larval specimens as well as pictures of the body at a deposition site. It was clear from the pictures that puparia were present at the site but no puparia had been collected. Therefore, Dr Hall was forced to give a limited report to the police, based on the only samples he had been sent (Hall, personal communication, 2017). Any errors will, at best, result in a report with limited value produced by the forensic entomologist and, at worst, result in an inaccurate minPMI estimation made by the forensic entomologist (Hall *et al.,* 2012). An example of the suggested protocol to follow at a crime scene is outlined by Amendt *et al* (2007). The problems of partial recovery

outlined by Lewis and Benbow (2011) assume that the protocol (Amendt *et al.*, 2007; Hall *et al.,* 2012) is followed; however a survey shows that in reality there are discrepancies in the practices that are followed (Magni *et al.,* 2013).

A comprehensive study of post-feeding larval dispersal is essential, mainly due to the contradictory nature of the current research and literature on this topic. This was highlighted in the literature review (Section 1.6).

1.7 Aims

This chapter has outlined the context of this PhD thesis in relation to the field of forensic entomology, while emphasising the limited nature of current research regarding post-feeding larval dispersal of blow flies. The literature review (Section 1.5) was divided up into the factors that affect, and are affected by, the post-feeding larval stage of blow fly development. Due to time constraints it was decided to examine the factors that affect post-feeding larval dispersal that are outlined in Sections 1.5.1 only. These factors were determined to be the most important to examine as they were the most widely commented on in the literature (Section 1.5.1) and had the most practical applications for optimising the location of post-feeding larvae and puparia at crime scenes (i.e. horizontal and vertical dispersal [Sections 1.5.1.1 and 1.5.1.2]).

The thesis aims to provide a more comprehensive understanding of post-feeding larval dispersal through the close examination of this under-studied stage of the blow fly lifecycle and therefore demonstrate the implications of the results for this field of science.

This thesis not only aims to produce data that will enable puparia and post-feeding larvae to be more easily located at body deposition sites, but also aims to understand the behaviour of post-feeding larvae prior to pupariation, which affects the final distribution of the puparia, i.e. where they will be located. Therefore, the more that is known about post-feeding larval behaviour and puparial distribution the more effective forensic practitioners, whether entomologists, SOCOs or CSIs will be at locating the oldest insect specimens associated with the colonisation of the body.

Specifically, the aims of this thesis were to determine:

- How the horizontal distance dispersed by post-feeding larvae was affected by the different substrates examined and any difference seen between species.

- How the vertical distance dispersed by post-feeding larvae was affected by the different substrates examined and any difference seen between species.
- Whether the active aggregation of post-feeding larvae prior to pupariation occurred in each species and in each substrate examined.
- The differences in the speed of post-feeding larvae between species was examined.

The overarching aim of this study is to produce a more comprehensive guide for forensic entomologists and SOCOs/CSIs for the collection of entomological evidence at deposition sites.

Chapter 2: Preliminary experiment

2.1 Introduction

The literature review revealed that the post-feeding larval dispersal stage of the blow fly has been under studied compared to other lifecycle stages and where research has been conducted, the results often conflict (Section 1.6). In order to determine the general methodology of this study and which factors of the post-feeding larval stage to focus on, it was decided to conduct two preliminary experiments. The intention of the preliminary study was to carry out experiments that were representative of some of the previous studies' methodologies, while also producing data considering multiple factors that may affect the post-feeding larval dispersal stage. The factors assessed during this experiment were: horizontal dispersal distances, vertical dispersal depth and the distribution of the sampled puparia to investigate aggregation of post-feeding larvae prior to pupariation and the dispersal direction of the movement of post-feeding larvae.

A review of the methodologies of the studies discussed in the literature review (Section 1.6) revealed some recurring themes. *Calliphora vicina* is the most common and prolific of all the species present at crime scenes in the UK (Smith, 1986; Szpila *et al.*, 2014; Hall *et al.*, 2015). It is a well-studied forensically important species of blow fly and this is reflected in current forensic entomology research (Arnott and Turner, 2008; Lewis and Benbow, 2011; Balme *et al.,* 2012; Frederickx *et al.,* 2014). *Calliphora vicina* was therefore the chosen species for this preliminary study. Different experimental apparatuses, of different shapes and dimensions have been used in past studies to investigate the many different aspects of the post-feeding larval dispersal stage (Table 2.1). A few studies have used circular apparatuses with varying diameters to study dispersing larvae (Table 2.1.). It was decided to use a 2.5 m (4.9 m^2) diameter circular apparatus in this study, i.e. much larger than apparatuses previously used which vary from 0.09 m to 0.5 m in diameter (Table 2.1). A larger apparatus was used because it was big enough to examine multiple factors of dispersing larvae (e.g. direction, aggregation prior to pupariation), but small enough to enable the data collection to take place in one day to ensure that all puparia were sampled at the same age and prior to adult emergence. Sawdust was used as a dispersal substrate during the preliminary experiment because of the ease of detecting dark puparia in the light substrate and due to the prevalence of its use in the literature (Table 2.1). The number of dispersing larvae introduced into the experimental apparatus for study varied greatly in the literature, from 1 to 350 larvae (Table 2.1). However, this present study introduced 1780 dispersing larvae in the first experimental run and then 150 dispersing larvae in the second run. It was decided to use a large and a small sample, in relation to the literature, to produce results

representative of both extremes. In order to ensure that most of the larvae had pupariated the apparatus was be sampled by hand in uniform sections ten days after the post-feeding larvae had been introduced (Andrade *et al.,* 2002; Reigada and Godoy, 2005).

The null hypothesis (H_0) for the preliminary experiment was that there would be aggregated numbers of puparia recovered from each sampled section. The alternative hypotheses were that there would either be even (i.e. equal numbers of puparia found in each sampled section) (H_1), or normal distribution (i.e. the number of puparia was concentrated in the original location, reducing towards the edge of the apparatus) of puparia recovered throughout the sampled sections of the circular apparatus (H_2).

Table 2.1: Summary of experiments to investigate larval dispersal. Relevant details of the experiments are shown: the species of blow fly, the number of larvae introduced, the area (m^2) and dimensions (m) of the experimental apparatuses, and the dispersal substrate used. After the dimension of each experimental apparatus the letter in brackets indicates the shape of the experimental apparatus, where: HR= horizontal rectangular shape (e.g. a tray), HC= horizontal circular shape (e.g. a petri dish) and VC= vertical circular shape (e.g. a vertical cylinder). L = length, W = width, D = diameter and H = height of each experimental apparatus.

Species	Number of larvae	Area/ m^2	Dimensions/m	Substrate	Reference
Chrysomya albiceps, Cochliomyia macellaria	60	0.2	1 L x 0.2 W (HR)	Sawdust	(Andrade *et al.*, 2002)
C. albiceps and C. macellaria (50:50 ratio)	120	0.2	1 L x 0.2 W (HR)	Sawdust	(Andrade *et al.*, 2002)
C. vicina	60-87	0.05	2 L x 0.025 W (HR)	Bare plastic	(Arnott and Turner, 2008)
C. vicina	100	0.05	2 L x 0.025 W (HR)	Bare plastic	(Arnott and Turner, 2008)
C. macellaria, Protophormia terraenovae	50	0.011	0.6 D x 0.15 H (VC)	Red clay soil	(Balme *et al.*, 2012)
Lucilia sericata	40	0.023	0.17 D (HC)	Agar, yeast and blood mix	(Boulay *et al.*, 2013)
L. sericata	1	0.0064	0.09 D (HC)	Wet sheet of paper	(Boulay *et al.*, 2013)
L. sericata	5	0.0014	Not stated	Soil	(Cammack *et al.*, 2010)
P. terraenovae	1	0.027	0.185 D (HC)	Agar	(Charabidze *et al.*, 2008)
Chrysomya megacephala	220, 294	0.9	3 L x 0.3 W (HR)	Sawdust	(Godoy *et al.*, 1995)
Chrysomya putoria	119,214	0.9	3 L x 0.3 W (HR)	Sawdust	(Godoy *et al.*, 1995)
C. macellaria	200,124	0.9	3 L x 0.3 W (HR)	Sawdust	(Godoy *et al.*, 1995)
C. megacephala	100	0.000035	0.2 D x 0.015 H (VC)	Vermiculite	(Gomes *et al.*, 2006b)
C. albiceps, L. cuprina	100	0.0094	0.3 D x 0.2 H (VC)	Vermiculite	(Gomes *et al.*, 2009)
C. albiceps, C. megacephala	50	0.049	0.25 D (HC)	Sawdust	(Gomes *et al.*, 2005)
C. albiceps	350	0.20	0.5 D (HC)	Sawdust	(Gomes and von Zuben, 2005)
C. albiceps, C. megacephala	120	0.2	1 L x 0.2 W (HR)	Sawdust	(Reigada and Godoy, 2005)
C. albiceps and C. megacephala (50:50 ratio)	120	0.2	1 L x 0.2 W (HR)	Sawdust	(Reigada and Godoy, 2005)

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2.2 Methodology

2.2.1 Establishing and rearing a colony of Calliphora vicina

Wild *C. vicina* adults were caught in November 2015, using Redtop® fly traps (Miller Methods, Pretoria, South Africa) baited with pork liver, in the wildlife garden of the Natural History Museum, London (51°29'46.9"N 0°10'43.2"W) (Ware *et al.,* 2016). Adult *C. vicina* were identified (after being chilled in the freezer for approximately 30 seconds) under a light microscope at up to 50 x magnification using the identification keys of Smith (1986). The adults were taken to the "colony room" in the SW tower of the Natural History Museum (Hofer *et al.*, 2017). They were transferred to 900 cm^3 insect rearing cages, consisting of a cubical frame of metal rods welded together inside of which was suspended a cage of fine netting with a single netting tube to enable access; the cages are bespoke and made to order (Figure 2.1). The adult flies were provided *ad libitum* with water, soaked in the central section of a sponge (3 x 15 cm) the ends of which were immersed in water through slits in the lid of a small tub (10 x 7 x 5 cm). Sugar was provided *ad libitum* as cubes, in a petri dish. The flies were exposed to natural diurnal light fluctuations via the large window in the room. Humidity was not controlled for, but the temperature was held constant by the building's air conditioning system at approximately 24°C, the recommended temperature for rearing conditions (Amendt *et al.,* 2007). Subsequent generations of *C. vicina* were maintained throughout the entire study period, with wild caught adults added each month to replenish the genetic diversity of the colony every month and maintain a colony of approximately 100 adults.

Figure 2.1: A netted 900 cm^3 cage containing adult *Calliphora vicina* blow flies and separate containers of water and sugar.

As the flies were wild caught the age of the adults was unknown. In order to induce gravidity, the adult flies were stimulated with the addition of approximately 20 g of pork liver (in a petri dish) for 24 hours, after which the liver was removed for another 24 hours and then new liver (approximately 20 g) was added for oviposition. Fresh liver was used as recommended by Richards *et al.* (2013). The eggs that were laid on the liver were transferred to a petri dish in a 1.3 litre Drennan Maggibox. These containers were placed in a LMS© 303 incubator that was set at 24 °C, while humidity was kept constant (around 45 % RH) with a water dish placed in the incubator. The larvae were fed with pork liver *ad libitum* to ensure there was no competition for food.

In order to ensure that the larvae used had not already started dispersing before the start of the experimental run, still feeding third instar larvae were used. When it was observed that at least one larva had left the food source (and was therefore beginning the dispersal phase), the larvae were deemed to be at the optimal stage of development to be used in an experimental run. The larvae were counted out manually and placed in each experimental treatment with the liver, that they were still feeding on (placed directly on the substrate surface).

2.2.2 Preliminary experiment methodology

A 2.5 m diameter (0.5 m height) plastic circular apparatus was filled with 8.4 kg of sawdust, approximately 5 cm deep (Figures 2.2.a and 2.2.b). Sawdust was used as a preliminary dispersal substrate as it provides a model pupariation medium (Andrade *et al.*, 2002). Due to a difference in density and composition, sawdust also offers a good comparison with different pupariation substrates, such as soil, that are subsequently used in this study (Chapter 3) (Greenberg and Szyska, 1984; Gomes *et al.*, 2006a; Mai and Amendt, 2012). The sawdust was bought from a local pet store, Pets Corner™ as it was guaranteed to be bacteria-free. Fresh sawdust was used for each experimental run to avoid contamination with ground marking signals (Boulay *et al.,* 2016) and for this same reason the apparatus was cleaned between uses with non-toxic washing-up liquid. The room was approximately 6 m^2 (2 x 3 m), with 2 doors and 2 windows (Figures 2.2.a, 2.2.b and 2.3).

Figures 2.2.a and 2.2.b: The set up of the 2.5 metre diameter circular apparatus. The apparatus was filled with sawdust, approximately 5 cm deep. A larval mass can be seen in the centre of the apparatus in Figure 2.2.b.

As was noted above, two preliminary experimental runs were conducted, the first with 1780 larvae and the second with 150 larvae. In each experimental run the larvae were placed in the centre of the apparatus simultaneously (Figure 2.2.b). Mean humidity was 54 % RH (min = 47.7 % and max = 61.6 %) and the temperature fluctuated with the external ambient temperature, the mean was 14.7 °C (min = 10.7 °C and max = 17.2 °C) within the room. The light in the room was uneven as there were two windows by one edge of the apparatus (Figures 2.2.a and 2.2.b). As only natural light was used, the light intensity in the room varied diurnally (sunrise 6:10-7:10 am and sunset 5:10-7:10 pm GMT ([L:D] 10:14 -13:11 h).

Once the introduced larvae had entered the puparial stage of development, i.e. after 10 days, the apparatus was sampled, by hand, in 64 approximately rectangular (~ 40 x 20 cm) sections using a hollow plastic box measuring 40 cm in length 20 cm in width and 10 cm in height (Figure 2.3). It is important to note that the sampled sections were not always exactly rectangular due to the circular shape of the sampled area (Figure 2.3). The numbers of puparia recovered from each section were recorded. The dataset was subsequently analysed using RStudio® and ArcGIS 10.1 (RStudio Team, 2016; ESRI 2018).

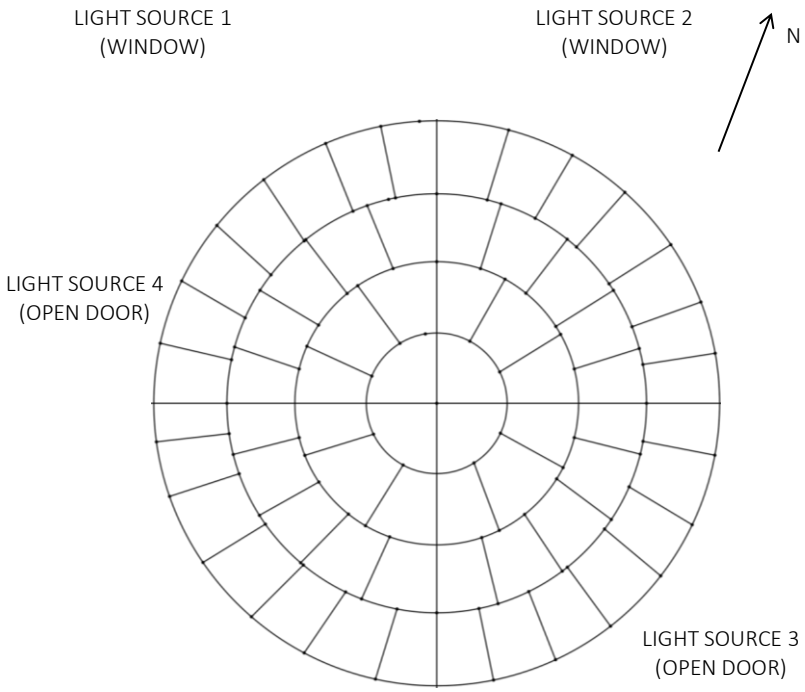

Figure 2.3: Arrangement of the sampled sections (40 x 20 cm) in the circular apparatus. The light sources indicate the direction of two windows (1 & 2) and two open doors (3 & 4) with windows on the other side of the doors. Not drawn to scale.

2.2.3 Data analysis

It was decided to use a mapping platform that is predominantly used in archaeological research, ArcGIS 10.1®, because the software allowed the production of simple visual representations of the data produced in a circular apparatus more clearly than other software packages. Additionally ArcGIS 10.1® can be used to perform statistical analyses (ESRI, 2018). The bar charts and the Chi-squared analyses were completed using RStudio® (RStudio Team, 2016)

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2.3 Results

2.3.1 First preliminary experimental run

The number of puparia recovered from each rectangular (40 x 20 cm) section is shown in Figure 2.4 (Section 2.2.3), while a Jenks (natural breaks) classification method (a data clustering method) was used to cluster the number of puparia found in each sampled section for visual representation (Figure 2.4).

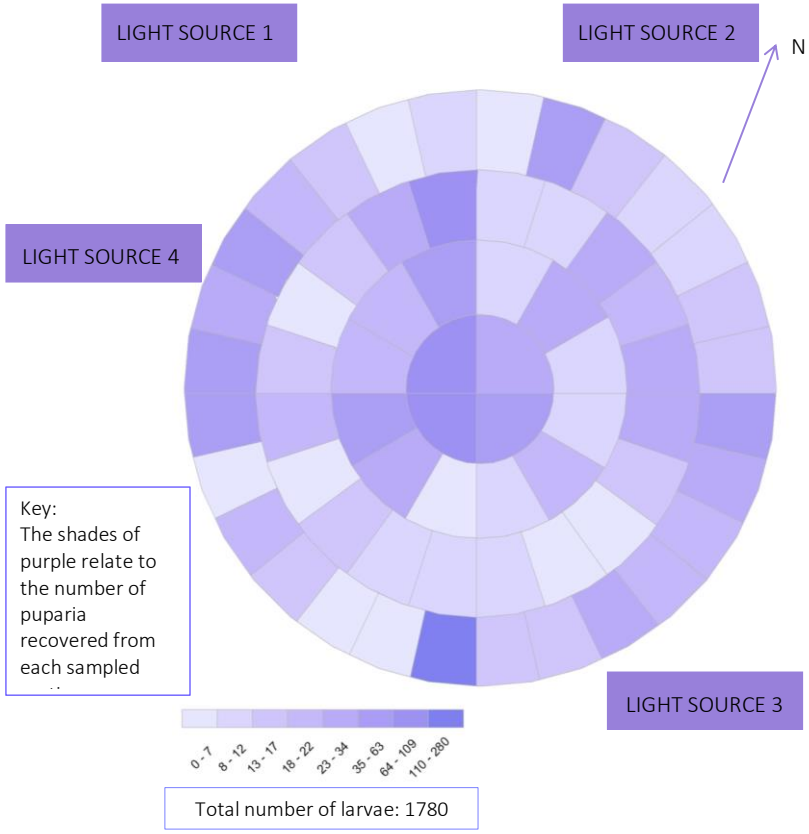

Figure 2.4: The number of puparia recovered from each (40 x 20 cm) segment from the first preliminary experimental run. The light sources show two windows (1 & 2) and two open doors (3 & 4) with windows on the other side of the doors.

To test the null hypothesis (i.e. that a random number of puparia would be recovered throughout the apparatus), the data was first analysed in frequency per sampled section (Figure 2.5). Visual inspection of the data suggests that there was no directional preference exhibited by the larvae prior to pupariation. However, 31 % of puparia were recovered from the top left quadrant, 17 % from the top right, 34 % from the bottom left and 18 % from the bottom right. This shows a preference for the left side of the arena (65 % larvae dispersed to the left and 35 % to the right). Moreover, a Chi-squared test, carried out in RStudio®, showed that the distribution was significantly not similar throughout the quadrants ($p < 0.0001$).

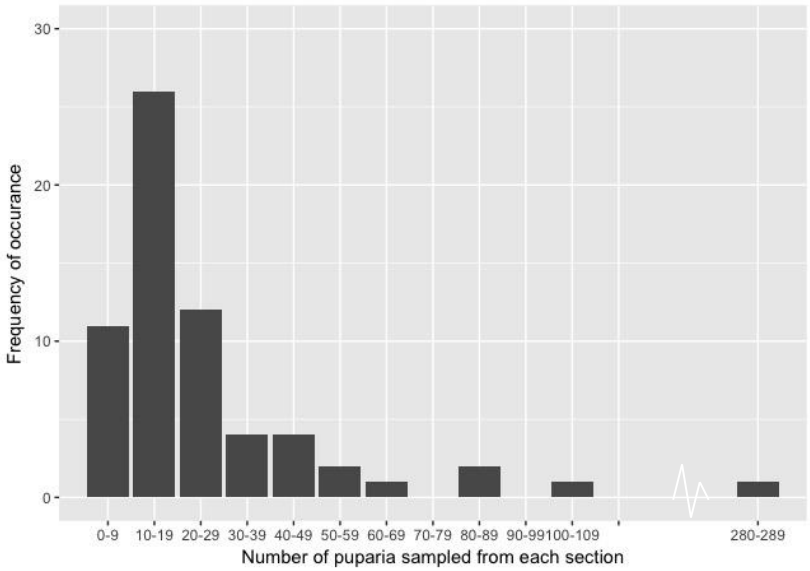

Figure 2.5: The frequency of the number of puparia recovered from each rectangular (40 x 20 cm) section in the first preliminary experiment. (n = 1780). Note: only one cluster containing more than 109 puparia was recovered.

Visual inspection of the data in Figure 2.5 clearly shows that the number of puparia recovered in each sampled section is not normally distributed and suggests that it is not even. The data appears to be positively skewed towards, favouring lower numbers of puparia recovered. To test whether the data was linear, a Chi-squared test was carried out in RStudio®. The results show that the distribution was highly significantly not even, $p < 0.0001$ (χ-squared = 3377.8, d.f. = 63). Therefore, both alternative hypotheses can be rejected and the null hypothesis accepted: random numbers of puparia were recovered. Aggregation of post-feeding larvae prior to pupariation is discussed in more detail in the following Chapters (Chapter 3 and Chapter 6). The puparia were recovered throughout the vertical column (5 cm) of the sawdust.

2.3.2 Second preliminary experimental run

The number of puparia recovered from each rectangular (40 x 20 cm) section is shown in Figure 2.6.

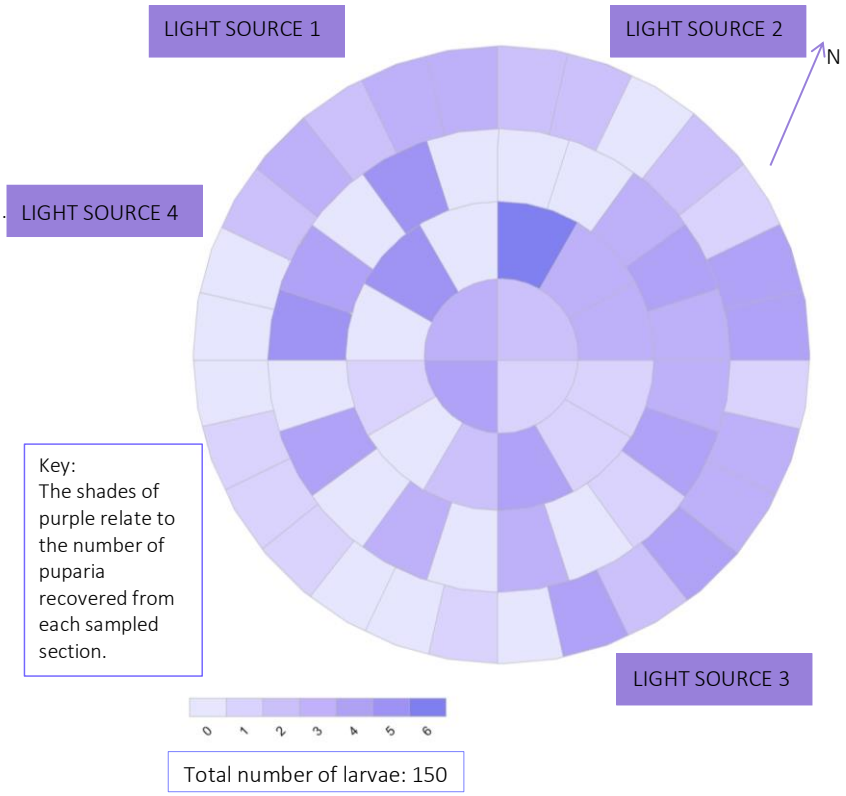

Figure 2.6: The density of puparia recovered from each (40 x 20 cm) segment from the second preliminary experimental run. The light sources show two windows (1 & 2) and two open doors (3 & 4) with windows on the other side of the doors.

The same hypotheses apply as in Section 2.3.1 apply in the present case: the null hypothesis, that there would be a random aggregation of variable numbers of puparia recovered in each sampled section; the alternative hypotheses were that there would either be an even or normal distribution of puparia recovered throughout the sampled sections of the circular apparatus. To test the null hypothesis, the data was analysed in frequency per sampled section (Figure 2.7). Visual inspection of the data suggests there was no directional preference exhibited by the larvae

prior to pupariation. However, 28 % of puparia were recovered from the top left quadrant, 31 % from the top right, 14 % from the bottom left and 27 % from the bottom right. This shows a slight disinclination for the bottom left side of the arena. A Chi-squared test, carried out in RStudio®, again showed that the distribution was significantly not similar throughout the quadrants (p = 0.041).

Again, after a visual inspection of the data in Figure 2.6, it is clearly not normally distributed. Figure 2.7 also suggests that the data were not linear, indicating aggregation. To test whether the data was even, a Chi-squared test was carried out in RStudio®. The results showed that the distribution was again, significantly not even, p = 0.01392 (X-squared = 90.197, d.f. = 63). Again, both alternative hypotheses can be rejected and the null hypothesis accepted: random numbers of puparia were recovered. The puparia were recovered throughout the vertical column (5 cm) of the sawdust.

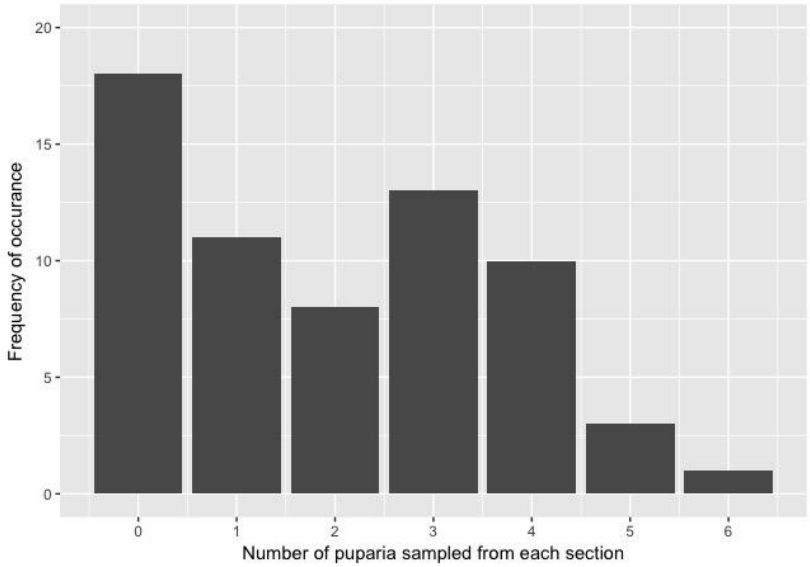

Figure 2.7: The frequency of the number of puparia recovered from each rectangular (40 x 20 cm) section in the second preliminary experiment. (n = 150).

2.4 Discussion

The preliminary experiment strongly suggests that dispersing larvae aggregate prior to pupariation. The Chi-squared test showed a statistically significant, non-linear, pattern of pupariation position. The *p*-value for the Chi-squared test for the first preliminary experimental run was highly significant ($p < 0.0001$). The *p*-value for the second experimental run was also significant ($p = 0.01392$), although less so. More experiments will need to be conducted to determine why the results showed a less statistically significant non-linear aggregation. While some studies suggest that dispersing larvae actively move away from other larvae in the pupariation substrate (Gomes and von Zuben, 2005), other studies agree that larval aggregation prior to pupariation does occur, although to differing extents between species (Godoy *et al.,* 1996; Lima *et al.,* 2009; Holmes *et al.,* 2013; Boulay *et al.,* 2016). Dispersing larvae tend to follow the trails of previous dispersing larvae (Arnott and Turner, 2008) and can detect other larvae in the pupariation substrate (Lima *et al.,* 2009). These preliminary results agree with some of the literature on this subject, as they suggest that larval aggregation is occurring. The preliminary experiment does not offer any explanation for the aggregation. It is most likely that this active aggregation is due to larvae passively producing chemical signals as they disperse, that are in turn interpreted by the subsequent dispersing larvae (Boulay *et al.,* 2013; 2015; 2016). Larval aggregation may also be positively thigmotactic, which is the physical response of an organism to a touch stimulus (Holmes *et al.*, 2013). Experiments need to be conducted to examine larval aggregation prior to pupariation, in particular to examine possible explanations for the aggregation (Chapter 3, 4 and 6).

While some studies suggest that there is a directional preference exhibited by dispersing blow fly larvae (Tessmer and Meek, 1996; Lewis and Benbow, 2011), many other studies suggest that there is none (Gomes and Zuben, 2005; Roux *et al.,* 2006; Arnott and Turner, 2008; Lima *et al.*, 2009). The results from the preliminary experiment as illustrated in Figures 2.4 and 2.6, suggest that there was no directional preference exhibited by the dispersing larvae. The puparia appear to be randomly distributed throughout the space of the circular apparatus. The position of the circular apparatus, and thus the sampling (Figures 2.4 and 2.6), in the unevenly lit room (Section 2.2.2) was constant for both experimental runs. When Figures 2.4 and 2.6 are visually compared to each other, there does not appear to be any obvious areas of the apparatus where correlating numbers of puparia were recovered. The Chi-squared tests conducted on the percentages of puparia recovered from the quadrants of each experimental run gave significant *p* values. There was directionality in dispersal shown in both experiments, but it was much stronger in the first experimental run (larval dispersal preference to the left). However, the directionality differed in

intensity and mean orientation between the two runs so, overall, there was no consistent preference shown by the dispersing larvae in the two runs in this apparatus. Moreover, there were no external factors that differed between each run (e.g. light intensity), to the author's knowledge, that could explain the difference in directionality exhibited by the dispersing larvae in each experimental run. Experiments will need to be conducted to investigate this further and repeated in the field to examine this area of research in more detail.

In both experimental runs puparia were recovered throughout the apparatus and up to the edge and therefore the larvae therefore dispersed up to 1.25 m, i.e. the radius of the apparatus (Section 2.2.2). The literature varies on the maximum horizontal distances that blow flies disperse, i.e. one study stated that blow flies can pupariate on the food source (Pohjoismäki *et al.,* 2010), while another suggested that they disperse up to 50 m before pupariating (Byrd and Castner, 2009). It is likely that the main reason for the discrepancy in the literature is that the post-feeding horizontal dispersal distance is species and environment (i.e. dispersal substrate) specific (Godoy *et al.,* 1996). The preliminary experiment used *C. vicina* only and therefore the following experiments reported in the next Chapter will include other UK blow fly species (*C. vomitoria*, *Lucilia sericata* and *Protophormia terraenovae*) to determine how far post-feeding UK blow fly larvae can move when less restricted by an apparatus, i.e. an apparatus that allows further horizontal dispersal.

It was also observed, during both experimental runs, that the puparia were found throughout the vertical "column" of sawdust. The sawdust was only 5 cm deep and therefore experiments will need to be conducted to determine how far *C. vicina* can disperse vertically. Although the literature suggests that most blow fly species pupariate in the top 5 cm of the pupariation substrate (Godoy *et al.,* 1995), studies have shown that when forced to, i.e. when buried artificially, adult blow flies can emerge from 50 cm deep in the soil (Balme *et al.,* 2012). It is likely that vertical dispersal depth is also species specific (Gomes *et al.*, 2005). Therefore, the experiments reported in Chapter 3 will investigate the vertical dispersal depth of different UK blow fly species, whilst examining vertical and horizontal dispersal in conjunction with each other, to determine how these factors interact. The literature also suggests that the dispersal substrate may affect the post-feeding dispersal of blow flies and therefore this factor is examined in Chapter 3.

Chapter 3: Horizontal and vertical dispersal of post-feeding larvae

Chapter 3 outlines the experiments that were conducted to examine the horizontal and vertical dispersal of post-feeding Calliphoridae larvae. Horizontal dispersal describes the movement of larvae in the horizontal plane and vertical dispersal describes larval movement in the vertical plane. This Chapter begins with introductory sections that explain horizontal dispersal (Section 3.1.1), vertical dispersal (Section 3.1.2) and simultaneous horizontal and vertical dispersal (Section 3.1.3). Each of those sections also include a list of hypotheses pertinent to the title of each Section (Sections 3.1.1.1, 3.1.2.1 and 3.1.3.1). Section 3.2 includes the methodologies used to examine horizontal and vertical dispersal: methodology used throughout all horizontal and vertical dispersal experiments (Section 3.2.1), methodology used to examine horizontal dispersal in a 6 m gutter (Section 3.2.2) and in the 1 m gutters (Section 3.2.3), methodology used to examine vertical dispersal in a pipe (Section 3.2.4) and the methodology used to examine horizontal and vertical dispersal simultaneously in a wooden box (Section 3.2.5). The results of each experiment type are presented in Section 3.3: results of the experiment conducted in the 6 m gutter (Section 3.3.1), results of the experiment conducted in the 1 m gutters (Section 3.3.2), results of the experiment conducted in the pipe (Section 3.3.3) and the results of the experiment conducted in the wooden box (Section 3.3.4). Section 3.4 is a discussion of all of the results presented in this Chapter.

3.1 Introduction

3.1.1 Horizontal dispersal

Horizontal dispersal describes larval movement in the horizontal plane, whether the dispersal occurs through a dispersal medium (e.g. soil or sawdust) or on the surface of the medium (e.g. waterlogged soil or concrete). One very important factor that may affect horizontal larval dispersal is the dispersal substrate (Arnott and Turner, 2008). This topic, however, has been under-studied. Some studies mention the dispersal substrate as a cause of certain dispersal behaviour (Arnott and Turner 2008; Lewis and Benbow 2011), most studies overlook this factor and one even stated that the water saturation of the substrate has no effect on the dispersing larvae (Miller, 1929). Miller (1929) tested the dispersal of *L. sericata* larvae (three days old, unknown instar or temperature that the larvae were reared at) on silk ribbon and stated that the saturation of the ribbon had no effect on the larval dispersal and therefore the type of dispersal substrate can be ignored. This paper has since been cited in the literature as a reference to argue

against substrate as a factor that affects post-feeding larval dispersal (Charabidze *et al.*, 2008). Despite the author's literature review above, there is some published data that has shown that the dispersal substrate affects dispersal distance, albeit that none of the experiments have examined different types of dispersal substrate, rather they examined the changes in only one dispersal substrate. For example, experiments that examined dispersal substrate density concluded that increased soil compaction and higher levels of water retention were associated with greater surface dispersal distances (Byrd and Castner, 2009; Lewis and Benbow, 2011). Some studies have expressed the opinion that the dispersal substrate could affects both horizontal and vertical dispersal distances, however, no studies were done (Dimou *et al.*, 2003; Gomes *et al.*, 2003; Cammack *et al.*, 2010).

Whether post-feeding larvae aggregate prior to pupariation is an area of behaviour that is only minimally discussed in the literature and the conclusions are conflicting. 'Aggregation' describes the active accumulation of larvae during different stages of development, and in this case, during the post-feeding dispersal stage, such that the resulting puparia become located in 'clusters' in the dispersal substrate. The aggregation of individuals of a species is well documented throughout the animal kingdom and applies to many fish (Brown and Laland, 2003), bird (Rubenstein and Lovette, 2007), mammal (Clutton-Brock, 2009) and insect species (Fewell, 2003). When there is an overall genetic gain in group living, aggregations are most likely to occur (Alexander, 1974). The benefits of these aggregations include: enhanced vigilance against predators (many sets of eyes and ears versus one), increased safety of migration, maximised inclusive fitness, and reduced impact of selection pressures (Alexander, 1974; Brown and Laland, 2003; Clutton-Brock, 2009). Essentially aggregations allow the population to function as a whole, displaying a complex set of behaviours not possible at the level of the individual organism (Parrish and Edelstein-Keshet, 1999). The same theories of the benefits of aggregations seen throughout the animal kingdom can be applied to the potential of the aggregation of post-feeding larvae prior to pupariation. It has been suggested that these benefits may also include a reduction in interspecific competition (Reigada and Godoy 2005; Lewis and Benbow 2011). Moreover, the aggregation of puparia has been seen to increase in the presence of parasitoids, conferring a 'safety in numbers' strategy, demonstrated by Cammack *et al.* (2010) who reported that *L. sericata* post-feeding larval aggregation increases prior to pupariation in the presence of the parasitoids wasp *Nasonia vitripennis*. The predation of calliphorid larvae by other calliphorid and muscid facultative predator larvae, as well as the interspecific larval competition for resources (i.e. food) is well known (Andrade *et al.*, 2002; Reigada and Godoy, 2005; Rosa *et al.*, 2006). However, interestingly, the level of aggregation of dispersing post-feeding larvae of *Cochliomyia macellaria* and *Chrysomya megacephala* has been shown to decrease in the presence of

Chrysomya albiceps larvae, a facultative predator (Reigada and Godoy, 2005). Thus, the interactions of predatory species and dispersing blow fly larvae is complicated.

The species chosen for the study present here were: *C. vicina, C. vomitoria, L. sericata* and *Protophormia terraenovae*. These species were selected as they are the most common species of forensically important Calliphoridae in the UK (Byrd and Castner, 2009). *Calliphora vicina* is the most common and prolific of all the species present at crime scenes (Smith, 1986; Szpila *et al.*, 2014; Hall *et al.*, 2015). *Calliphora vomitoria* (Smith, 1986; Frederickx *et al.*, 2012; Szpila *et al.*, 2014) and *L. sericata* (Smith, 1986; Frederickx *et al.*, 2012; Szpila *et al.*, 2013) are also very common at UK crime scenes. *Protophormia terraenovae* (Smith, 1986) is found throughout the UK and is present in around 8 % of cases that are worked on by a forensic entomologist (Hall and Mactaggart, 2018). This species was also chosen because it is more often described as a species that does not disperse from the body at a crime scene, but pupariates on or very close to the body itself (Erzinçlioğlu, 1996; Gennard, 2007). *Protophormia terraenovae* therefore, was determined as a good species in comparison to other species' post-feeding larvae dispersal. Four different dispersal substrates were examined in the experiments that are described: Natural garden topsoil, commercial topsoil, sawdust and bare plastic. The experiments were conducted to determine whether there were differences in the horizontal dispersal distances with each different substrate, whether different species of blow fly larvae reacted differently to the different dispersal substrates and whether the overall puparial distribution of each species of larvae differed with each substrate.

3.1.1.1 Horizontal dispersal hypotheses

The experiments reported in this Chapter were conducted in order to examine a) how far the horizontal post-feeding larval distance dispersed and; b) whether the distribution of the puparia recovered from the experimental apparatus was affected by substrate. The experimental apparatuses used to examine horizontal dispersal were plastic gutters (Section 3.2.2 and Section 3.2.3), essentially plastic cylinders cut in half horizontally and capped at each end, such that larvae were able to move freely throughout the apparatus and different substrates could be added.

Thus, for each of the experimental runs conducted there were multiple hypotheses that were tested, firstly looking at the overall distance dispersed and distribution pattern of the puparia recovered during each experiment to determine if there were any differences that occurred due to the dispersal substrate:

H_0: There would be no difference in distribution pattern of the puparia or overall distance dispersed by the post-feeding larvae as a result of the different dispersal substrates used.
H_1: There would be a difference in the distribution pattern and/or the overall distance dispersed by the post-feeding larvae shown in at least one of the dispersal substrates, when compared to the others.

The second set of hypotheses examined distribution of the puparia recovered from the gutter:

H_0: The distribution of the puparia recovered from the gutter would be even, i.e.: the same number of puparia would be recovered from each section.
H_1: There would be a normal distribution of puparia recovered, most likely skewed to the section of the gutter where the post-feeding larvae were introduced.
H_2: There would be a random distribution, i.e. there would be no discernible pattern to the number of puparia recovered from each section.
The third set of hypotheses that were tested examined the differences shown between species:

H_0: There would be no difference in the overall distance dispersed by the post-feeding larvae or the distribution pattern of the puparia of each species.
H_1: There would be a significant difference in the overall distances dispersed, but no difference in the distribution pattern of the puparia of each species.
H_2: There would be no difference in the overall distances dispersed, but a difference in the distribution pattern of the puparia of each species.
H_3: There would be a difference in the overall distances dispersed and in the distribution pattern of the puparia of each species.

3.1.2 Vertical dispersal

As outlined in Sections 1.2 and 3.1.1, the primary task of a forensic entomologist at a deposition site is to locate the oldest specimens associated with the body in order to subsequently estimate minPMI (Amendt *et al.*, 2004). Once post-feeding larvae have dispersed to find a suitable pupariation site, where possible the larvae then burrow into the dispersal substrate prior to pupariation (Gomes *et al.*, 2006a). As discussed in Section 3.1.1 the dispersal substrate may affect horizontal larval dispersal and thus, may also affect vertical larval dispersal. There is little literature concerning the burial depth of post-feeding larvae and where the literature is present it does not always agree (Section 1.6.1.2). For example, burial depths of up to 32 cm deep have

been recorded for *Chrysomya megacephala* in sawdust shavings (Gomes *et al.*, 2005), although the majority of sources agree that the puparia of most species of blow fly can usually be found naturally within the top 5 cm of the burial substrate (Godoy *et al.*, 1995; Gennard, 2007). The experimental apparatus used here to examine vertical dispersal was a plastic pipe (Section 3.2.4), sawn into sections and reconstructed such that it allowed larval movement throughout the apparatus unimpeded at the commence of the experimental run and then allowed the examination of the puparia recovered from each section at the end of each experimental run.

The species chosen for this study was *C. vicina*, as it is one of the most common species of forensically important Calliphoridae in the UK (Byrd and Castner, 2009) and the most common and prolific of all the species present at crime scenes (Smith, 1986; Szpila *et al.*, 2014; Hall *et al.*, 2015). *Calliphora vomitoria, L. sericata* and *P. terraenovae* were not used in this experiment due to time constraints.

3.1.2.1 Vertical dispersal hypotheses

The first set of hypotheses tested examined the dispersal depth capabilities of the post-feeding larvae:

H_0: The majority of puparia would be recovered from the top 5 cm of the soil, as is often cited in the literature as the normal burrowed depth of post-feeding larvae.
H_1: The majority of puparia would be recovered from over 5 cm deep.

The second set of hypotheses examined the distribution pattern of the burrowed larvae:

H_0: The distribution of the puparia recovered from the pipe would be even, i.e. the same number of puparia would be recovered from each section.
H_1: The distribution of the puparia recovered would be positively skewed, the majority of puparia recovered closer to the origin (the top of the pipe), but some recovered deeper.
H_2: There would be a random distribution, i.e. no discernible pattern to the number of puparia recovered from each section.

The third set of hypotheses examined differences between the two dispersal substrates examined:

H_0: The distribution of the puparia recovered would be the same for both dispersal substrates tested.
H_1: There would be a difference in the distribution patterns between the two substrates examined.

3.1.3 Simultaneous horizontal and vertical dispersal

A third type of experimental set-up examined horizontal and vertical dispersal simultaneously (Section 3.2.4 and Section .3.3.4).

3.1.3.1 Simultaneous horizontal and vertical dispersal hypotheses

The final set of hypotheses pertained to the simultaneous horizontal and vertical experiment only (Section 3.2.5 and Section 3.3.4). These hypotheses examined the horizontal and vertical distribution of the puparia recovered:

H_0: There would be an even distribution of puparia throughout the experimental apparatus.
H_1: More puparia would be found throughout the upper part of the apparatus, i.e. post-feeding larvae would favour horizontal dispersal.
H_2: The puparia would be recovered in a 'column' vertically below the origin of the post-feeding larvae, i.e. the larvae would favour vertical dispersal.

3.2 Methodology

3.2.1. Horizontal and vertical dispersal experiments

Colonies of *C. vicina, C. vomitoria, L. sericata and P. terraenovae* were established and reared as outlined in Section 2.2.1. These species were used as they have been identified as the most important UK calliphorid species of forensic importance (Section 3.1.1).

Four different substrates were used during the horizontal dispersal experiments (Note: not all substrates were used during all experiments):

1. Topsoil from the Natural History Museum was used because it most closely represented an outdoor deposition site environment and was representative of an English woodland environment, containing leaf litter and natural soil fauna, including microbes. This topsoil

was acquired from the Wildlife Garden at the Natural History Museum in London (51°29'46.9"N 0°10'43.2"W) (Ware *et al.* 2016).

2. The commercial topsoil was Multi-Purpose Topsoil purchased from a shop in the Wickes© DIY chain.
3. Sawdust was used as a dispersal substrate as it provided a model pupariation medium, frequently used in forensic entomology research, and offered a good comparison with different pupariation substrates used elsewhere (Greenberg and Szyska, 1984; Gomes *et al.,* 2006b; Mai and Amendt, 2012). The sawdust was bought from a local pet store, Pets Corner™.
4. The bare gutter (no substrate) was used to represent an indoor scenario with a smooth floor surface, such as smooth concrete.

After each experimental run the equipment used was emptied of the dispersal substrate used and cleaned with a non harmful washing up liquid. Once a dispersal substrate was used in an experimental run, it was not reused. Lux light levels were measured throughout all of the experimental apparatuses, using a Heavy Duty Lightmeter (HD450) with the sensor aimed vertically upwards (Table 3.1 [Section 3.2.2]; Table 3.3 [Section 3.2.3]; Table 3.5 [Section 3.2.4] and Table 3.7 [Section 3.2.5]). A factory calibrated digital data logger, that included a thermometer, (Tinytag Plus 2® Gemini Data Loggers), set to record hourly on the hour, was placed in the experimental room (colony room) to record temperature and humidity. All data were recorded and analysed using RStudio®. The ratio of variance to the mean ($s^2/\bar{x}$) is a statistical test that has been used to determine levels of larval clustering in a Dipteran dataset (Butlin *et al.*, 1984). A ratio greater than one suggests that the data is clumped, while a ratio of one suggests that the data follows a Poisson distribution (Butlin *et al.*, 1984). The data collected was all tested using the ratio of variance to the mean test to examine levels of clustering.

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3.2.2 Horizontal dispersal experiment conducted in the six metre gutter

A 6 m long plastic gutter (600 cm x 10 cm x 10 cm) was constructed in 3 sections, each of 2 m, to examine the distances dispersed by the larvae (Figure 3.1). There was a single gutter set-up, such that each experimental run was conducted separately.

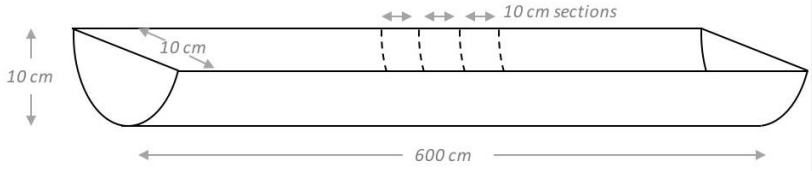

Figure 3.1: The dimensions of the 6 m plastic gutter. (Not drawn to scale).

The gutter was laid on the floor of a temperature-controlled laboratory (20 °C) and then filled with one of two different substrates: Wildlife Garden topsoil or sawdust. A third set-up was used to look at horizontal dispersal in an indoor setting; the bare plastic of the gutter itself, with no added substrate.

The substrate was added to the gutter to a depth of approximately 6 cm for the entire length of the gutter (Figure 3.2). Post-feeding larvae (determined by the process described in Section 2.2.1) were added to one end of the gutter. The number of larvae added per run varied, depending on the number reared to the post-feeding stage at that time. The number of larvae reared at a time varied greatly and therefore the decision was made to use the number available during each experimental run (the efficacy of this is shown in Section 3.3.1). The number of larvae added to each experimental run varied from 100 to 1200 and is shown in Section 3.3.1. The gutter was then covered with cardboard (Figure 3.3) that concealed the entire length of the gutter to ensure consistent low light levels throughout the inside of the gutter (Table 3.1).

Figure 3.2: The 6 m plastic gutter *in situ*, filled with 6 cm deep topsoil.

Figure 3.3: The gutter with a cardboard roofing structure covering the entire length to ensure consistent low light levels.

Table 3.1: Lux light level measurements taken once a day over a 5 day period. The results are shown for measurements taken at 4 locations throughout the length of the gutter without the cardboard cover and with the cardboard cover. All measurements are in lux.

Location	Without cardboard cover		With cardboard cover
	Mean	Range	Mean
0 cm	99	87-111	0
200 cm	162	141-189	0
400 cm	151	122-191	0
600 cm	21	19-22	0

After ten days, once the introduced larvae had entered the puparial stage of development, the gutter was sampled by hand in 60 sections, each of 10 cm length (Figure 3.1). The numbers of puparia recovered from each section were recorded.

A total of 12 experimental runs were conducted using a combination of one of two species and one of the three substrates (Table 3.2).

Table 3.2: Summary of the experimental runs conducted using a 6 m long gutter, showing the number of runs conducted using each species, substrate and the dates that the experiments were conducted on.

Species	Substrate	Number of experimental runs	Dates
C. vicina	Commercial soil	3	10/02/16, 11/03/16, 15/04/16
C. vicina	Bare	3	11/05/17, 04/07/17, 14/08/17
C. vicina	Sawdust	3	27/06/16, 07/07/16, 19/10/17
P. terraenovae	Sawdust	3	13/11/17, 22/12/17, 13/02/18

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3.2.3 Horizontal dispersal experiment conducted in the one metre gutters

Six 1 m long plastic gutters were constructed to examine the aggregation of post-feeding larvae prior to pupariation (Figure 3.4.a). The exact dimensions of the gutters were 100 x 10 x 10 cm (Figure 3.4.b).

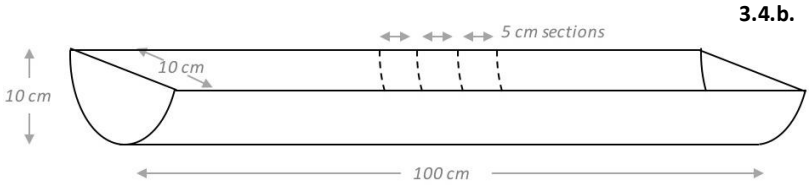

Figure 3.4.a: The six 1 m plastic gutters *in situ*. **Figure 3.4.b.:** Dimensions of the gutters.

The gutters were laid on the floor of a temperature-controlled laboratory and then filled with one of three different substrates: Wildlife Garden topsoil, commercial topsoil or sawdust (Section 3.2.1). A fourth set-up was used to look at horizontal dispersal in an indoor setting, i.e. the bare plastic of the gutter itself, with no added substrate (Section 3.2.1).

The substrate was added to each gutter to a depth of approximately 6 cm for the entire length of the gutter (Figure 3.4.a). Post-feeding larvae (determined as such by the same process as set out in Section 2.2.1) were added to the middle of each gutter. The number of larvae added per run varied, depending on the number reared to the post-feeding stage at that time. The number of larvae added to each experimental run varied from 30 to 160 and is shown in Section 3.3.2. The gutters were then covered with cardboard that concealed the entire length of the gutter to ensure low light levels were consistent throughout the inside of the gutter (Table 3.3).

Table 3.3: Lux light level measurements taken once a day over a 5 day period. The results are shown for measurements taken at 3 locations throughout the length of the gutter without the cardboard cover and with the cardboard cover. All measurements are in lux.

Location	Without cardboard cover		With cardboard cover
	Mean	Range	Mean
0 cm	210	206-214	0
50 cm	213	205-222	0
100 cm	280	271-294	0

After ten days, once the introduced larvae had entered the puparial stage of development, the gutters were sampled by hand in 20 sections, each of 5 cm length (Figure 3.4.b.). The gutters were sampled in 5 cm sections (in comparison to the 10 cm sections of the 6 m gutter runs [Section 3.2.2]) so that any aggregations would not be overlooked. The numbers of puparia recovered from each section were recorded.

A total of 56 experimental runs were conducted using a combination of one of the four species and one of the four substrates (Table 3.4).

Table 3.4: Summary of the experimental runs conducted using a 1 m long gutter, showing the number of runs conducted using each species, substrate and the dates that the experiments were conducted on.

Species	Substrate	Number of experimental runs	Dates
C. vicina	Wildlife Garden soil	7	05/05/16, 23/05/16
C. vicina	Sawdust	12	15/06/16, 27/06/16
C. vicina	Bare	11	04/06/16, 11/05/17
C. vicina	Commercial soil	5	14/08/17
L. sericata	Bare	5	03/07/17
L. sericata	Commercial soil	5	14/07/17
C. vomitoria	Commercial soil	5	17/03/17
P. terraenovae	Sawdust	6	22/12/17

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3.2.4 Vertical dispersal experiment conducted in a pipe

A 72 cm tall plastic pipe, with a diameter of 8 cm, was constructed to examine the vertical depth dispersed by the larvae (Figure 3.5.a and 3.5.b).

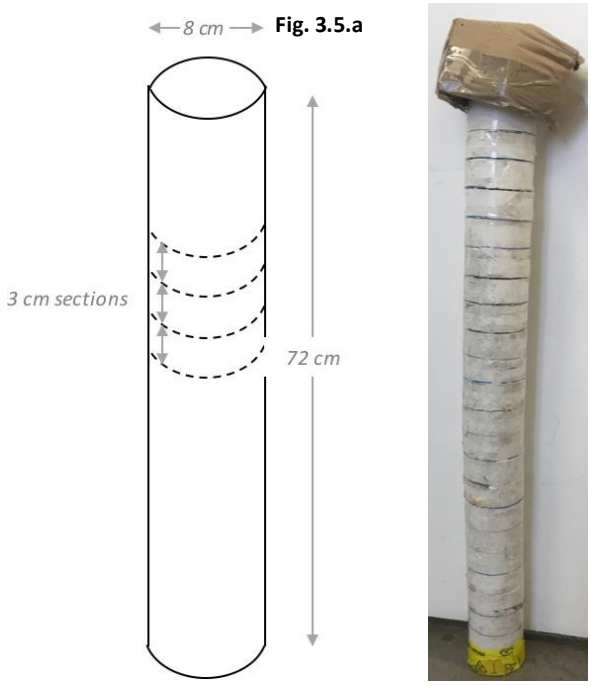

Figure 3.5.a: The dimensions of the plastic pipe. **Figure 3.5.b:** The 72 cm plastic pipe *in situ*. The pipe is divided into 24 x 3 cm thick sections. The cardboard roof structure is shown, covering the top of the pipe, to ensure low light levels inside the pipe.

The 3 cm sections of pipe were taped together (using Duct Tape) prior to the substrate being added. Once *in situ* the pipe was filled with one of two different substrates: commercial topsoil or sawdust substrate (Section 3.2.1). The substrate was added to the pipe such that the top 6 cm contained no substrate, to ensure that none of the larvae escaped. The base of the pipe was then tapped on the ground gently to aid settling by gravity, which resulted in an average density of 0.066 g/cm^3 of sawdust and 0.47 g/cm^3 of commercial topsoil. The pipe was then topped with cardboard to ensure that no light was present inside the pipe (Figure 3.5.b; Table 3.5). Post-feeding larvae (determined as such by the same process outlined out in Section 2.2.1) were added to the top of the pipe. The number of larvae added per run varied, depending on the number reared to the post-feeding stage at that time. The number of larvae added to each experimental run varied from 50 to 1200 and is shown in Section 3.3.3.

Table 3.5: Lux light level measurements taken once a day over a 5 day period. The results are shown for measurements taken from inside the pipe, above the substrate without the cardboard cover and with the cardboard cover. All measurements are in lux.

Location	Without cardboard cover		With cardboard cover
	Mean	Range	Mean
On top of the substrate inside the pipe	198	190-204	0

After ten days, once the introduced larvae had entered the puparial stage of development, the 3 cm pipe sections were separately sampled (Figure 3.5.a. and 3.5.b.). The taped 3 cm sections of pipe were untaped section-by-section starting at the top and in this way the number of puparia recovered from each section were recorded with relative ease.

A total of six experimental runs were conducted using *C. vicina* and one of the two substrates (Table 3.6).

Table 3.6: Summary of the experimental runs conducted using a pipe, showing the number of runs conducted using each species, substrate and the dates that the experiments were conducted on.

Substrate	Number of experimental runs	Dates
Commercial soil	3	07/11/16, 03/02/17, 28/03/17
Sawdust	3	27/06/16, 16/07/16, 19/10/17

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3.2.5 Simultaneous horizontal and vertical dispersal experiment conducted in a wooden box

Two wooden 'boxes' were constructed to simultaneously examine the vertical and horizontal dispersal of post-feeding larvae prior to pupariation (Figures 3.6.a, 3.6.b and 3.6.c). The dimensions of the 'boxes' were 100 cm width x 50 cm height x 5 cm depth (Figure 3.6.a). The 'boxes' were constructed such that the introduced larvae were able to disperse unimpeded in two planes, horizontally and vertically.

Fig. 3.6.a

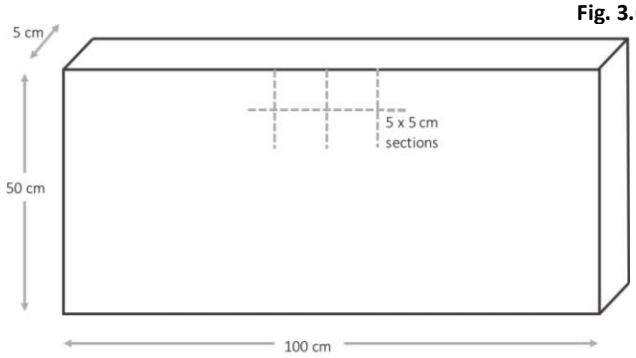

Fig. 3.6.b

Fig. 3.6.c

Figure 3.6.a: The dimensions of the wooden 'box' (not to scale). **Figure 3.6.b:** The 'box' viewed from the side. **Figure 3.6.c:** Aerial view of the 'box'.

The apparatuses were then filled with three different substrates: Wildlife Garden topsoil, commercial topsoil or sawdust.

The substrate was added to the boxes such that they were filled almost to the top, leaving a 5 cm gap to ensure that no larvae were able to escape (Figure 3.6.c.). Post-feeding larvae (determined as such by the same process as set out in Section 2.2.1) were added to the middle of each 'box' (on top of the substrate). The number of larvae added per run varied, depending on the number reared at that time. The number of larvae added to each experimental run varied from 100 to 300 and is shown in Section 3.3.4. The top of the 'boxes' were then covered with cardboard that concealed the entire length of the 'box' to ensure low light levels were constant throughout the inside of the box (Table 3.7).

Table 3.7: Lux light level measurements taken once a day over a 5 day period. The results are shown for measurements taken at 3 locations throughout the length of the width of the 'box', on top of the substrate, without the cardboard cover and with the cardboard cover. All measurements are in lux.

Location	Without cardboard cover		With cardboard cover
	Mean	Range	Mean
0 cm	130	119-137	0
50cm	115	107-120	0
100 cm	200	195-203	0

The 'boxes' were constructed such that one side could be unscrewed and removed. After ten days, when the introduced larvae had entered the puparial stage of development, the 'boxes' were sampled. To facilitate sampling, the 'box' was carefully placed on the floor on its side and the upper side was removed (Figure 3.7.a). Sampling was by hand, in 5 x 5 cm sections (Figure 3.7.a and 3.7.b). The numbers of puparia recovered from each section were recorded.

Fig. 3.7.a

Fig. 3.7.b

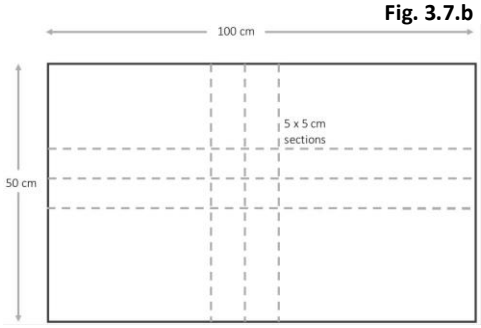

Figure 3.7.a: Wooden 'box' shown open, after it has been placed on its side and one side of the 'box' has been removed, leaving the bulk of the substrate (in this case soil) in the rest of the box (upper half of the figure). **Figure 3.7.b:** The inside of the 'box' when it is on its side, the dimensions of the 'box' and the sampling sections are shown (not to scale).

A total of six experimental runs were conducted using *C. vicina* and one of the two substrates (Table 3.8).

Table 3.8: Summary of the experimental runs conducted using a wooden box, showing the number of runs conducted using each species, substrate and the dates that the experiments were conducted on.

Substrate	Number of experimental runs	Dates
Commercial soil	3	30/09/16, 09/02/17, 22/03/17
Sawdust	3	19/10/17, 19/11/17, 13/12/17

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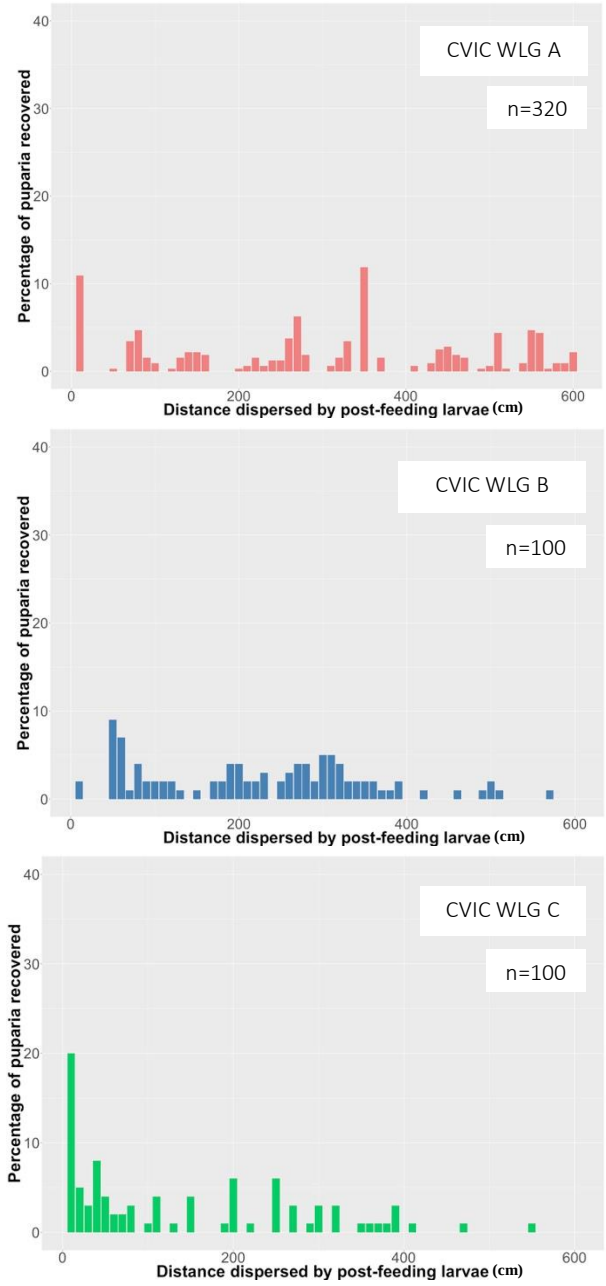
3.3 Results

Initially it was intended to use topsoil from the Wildlife Garden for all of the experiments that used topsoil. However, commercial, shop bought, topsoil was used for the majority of experiments because the topsoil from the Wildlife Garden was not homogenous and therefore was difficult to sample. Commercial soil was homogenous and was considered to be an acceptable compromise, combining the representation of a 'real-life' scenario with the greater accuracy of sampling. There was an in increase in the time spent sampling the Wildlife Garden topsoil, in comparison to the commercial topsoil (the 6 m bare gutter took ~ 20 minutes to sample, the sawdust and commercial topsoil substrates took ~ 1 hour and the Wildlife Garden soil took up to ~ 3 hours). Due to the limited time available and the number of experimental runs conducted, it was decided to use the substrate that increased sampling efficiency.

3.3.1 Six metre horizontal dispersal experiment

This Section provides an account of nine experimental runs conducted using *C. vicina* post-feeding larvae and three runs using *P. terraenovae* post-feeding larvae.

C. vicina larvae were used for the first three sets of experimental runs. The dispersal substrate used for the first set of experimental runs was topsoil collected from the Wildlife Garden at the Natural History Museum in London (Ware *et al.*, 2016). The results of each of these experiments are presented in Figures 3.8.a-c. There was no dispersal substrate used for the second set of runs, so that the larvae were dispersing directly on the bare plastic of the gutter. The results of each of these runs are presented in Figures 3.9.a-c. Sawdust was used as a dispersal substrate for the third set of runs and the results are presented in Figures 3.10.a-c. The final set of runs were conducted using *P. terraenovae* larvae with sawdust as a dispersal substrate. Again, different numbers of individuals were added to each experimental run and the results are presented in Figures 3.11.a-c.



Figures 3.8.a-c: The percentage of puparia recovered from each 10 cm section (60 sections in total) from each experiment conducted in the 6 m gutter. 'CVIC' = *Calliphora vicina* and 'WLG' = Wildlife Garden soil substrate. The number of individuals used in each experiment is shown on each graph by 'n'.

Table 3.9: Summary of the ratios of variance to the mean conducted on each experimental run, where a score > 1.0 indicates aggregation and a score closer to 1 suggests a Poisson distribution. 'CVIC' = *Calliphora vicina* and 'WLG' = Wildlife Garden soil substrate.

	Number of post-feeding larvae	Ratio of variance to the mean
CVIC WLG A	320	10.98
CVIC WLG B	100	2.09
CVIC WLG C	100	6.09

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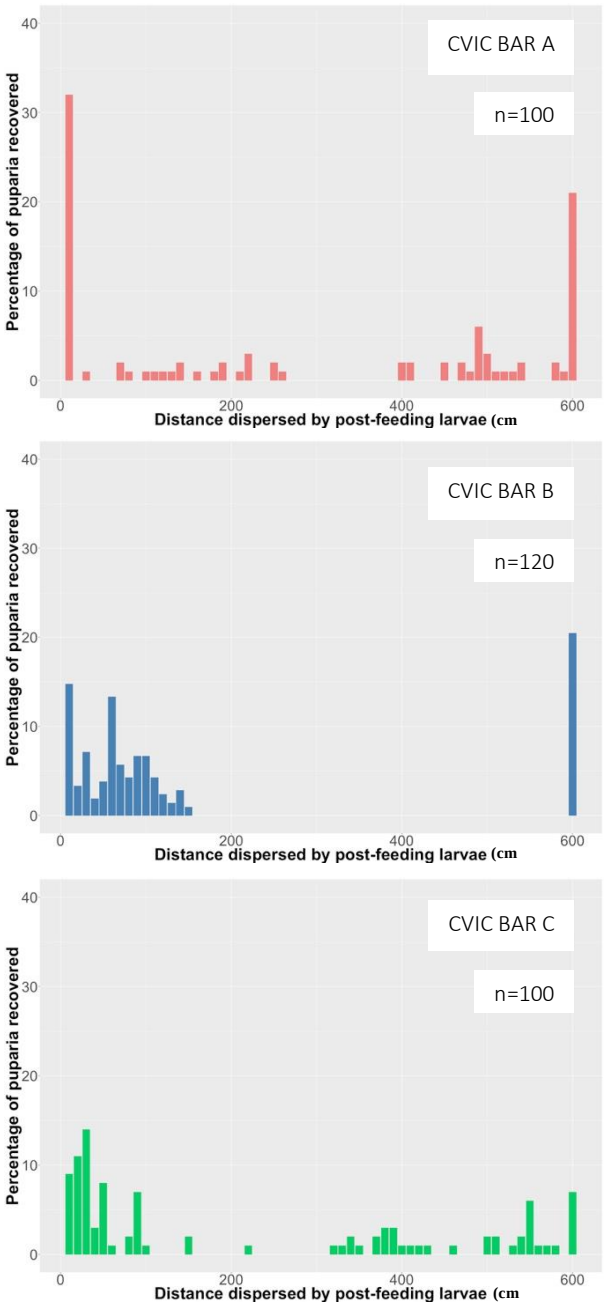
The distributions of recovered puparia in CVIC WLG A-C were very similar and in an oscillating pattern and in all instances puparia were recovered throughout the length of the gutter. However, the majority of puparia (> 50 %) were recovered from within the first 3 m of the gutter. The ratios of variance to the mean (Table 3.9) conducted on the results from each experimental run produced a ratio much greater than one, suggesting that the distribution of puparia recovered was highly clumped. These results may provide evidence of aggregation prior to pupariation. These results are examined in more detail in the following Sections (Sections 3.3.1.1 and 3.3.1.2) and discussed at the end of this Chapter (Section 3.4).



Figures 3.9.a-c: The percentage of puparia recovered from each 10 cm section (60 sections in total) from each experiment conducted in the 6 m gutter. 'CVIC' = Calliphora vicina and 'BAR' = bare (no substrate). The number of individuals used in each experiment is shown on each graph by 'n'.

Table 3.10: Summary of the ratios of variance to the mean conducted on each experimental run, where a score > 1.0 indicates aggregation and a score closer to 1 suggests a Poisson distribution. 'CVIC' = *Calliphora vicina* and 'BAR' = bare (no substrate).

	Number of post-feeding larvae	Ratio of variance to the mean
CVIC BAR A	100	14.31
CVIC BAR B	120	19.30
CVIC BAR C	100	5.08

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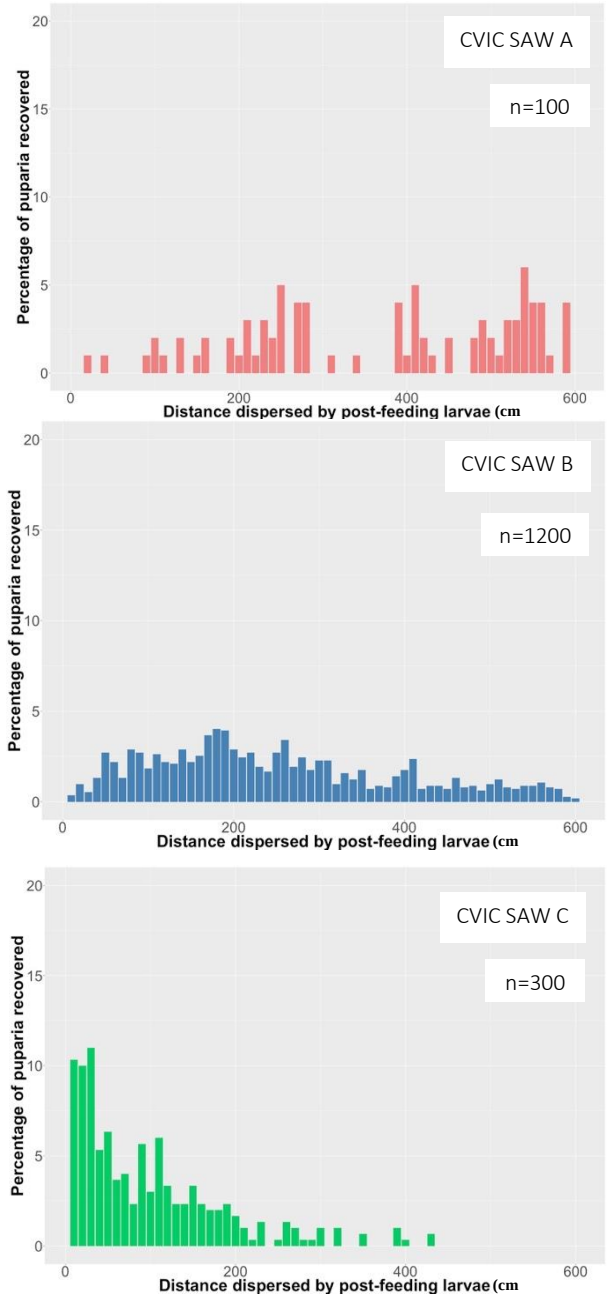
The results of CVIC BAR A-C are also similar to each other; a large percent of puparia (> 40%) were recovered from either end of the gutter and > 50 % from within the first 3 m of the gutter. The ratios of variance to the mean (Table 3.10) conducted on the results from each experimental run produced a ratio much greater than one, suggesting that the distribution of puparia recovered was highly clumped. These results may provide evidence of aggregation prior to pupariation. These results are examined in more detail in the following Sections (Sections 3.3.1.1 and 3.3.1.2) and discussed at the end of this Chapter (Section 3.4).



Figures 3.10.a-c: The percentage of puparia recovered from each 10 cm section (60 sections in total) from each experiment conducted in the 6 m gutter. 'CVIC' = *Calliphora vicina* and 'SAW' = sawdust substrate. The number of individuals used in each experiment is shown on each graph by 'n'.

Table 3.11: Summary of the ratios of variance to the mean conducted on each experimental run, where a score > 1.0 indicates aggregation and a score closer to 1 suggests a Poisson distribution. 'CVIC' = *Calliphora vicina* and 'SAW' = sawdust substrate.

	Number of post-feeding larvae	Ratio of variance to the mean
CVIC SAW A	100	1.85
CVIC SAW B	1200	6.35
CVIC SAW C	300	12.40

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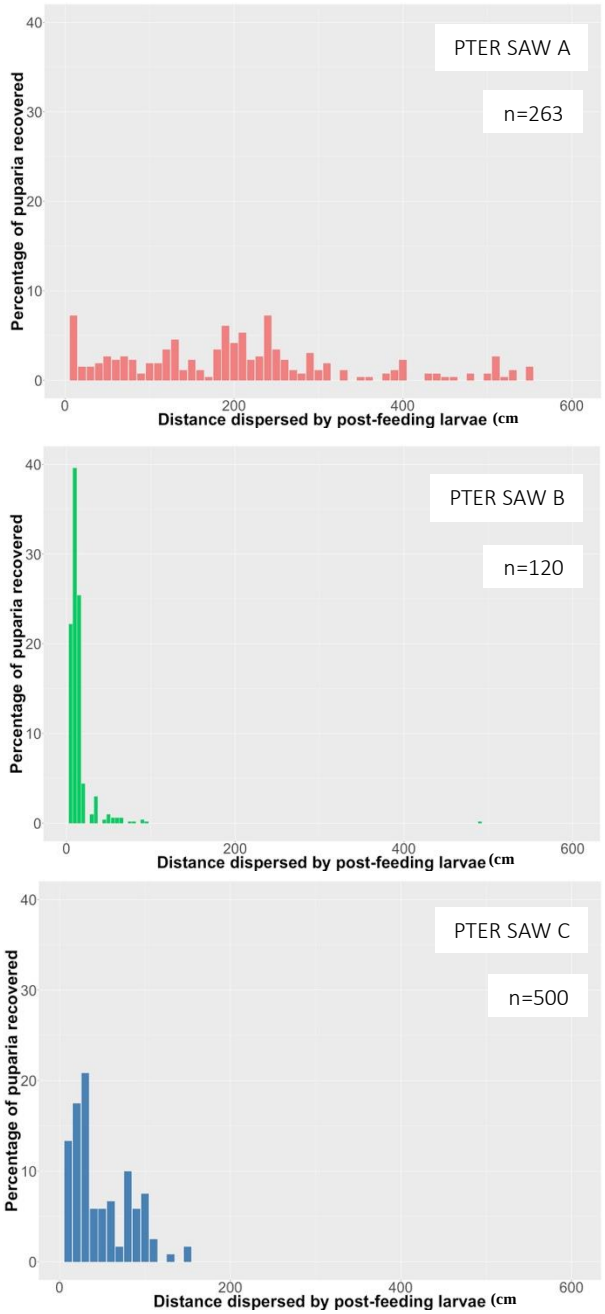
The results of CVIC SAW A and B show a similar pattern and appear to be more consistent with the results of CVIC WLG A-C, where the puparia were recovered throughout the length of the gutter and in an oscillating pattern. The majority of puparia (> 50 %) were recovered from within the first 3 m of the gutter for CVIC SAW B and C, and within the first 4 m for CVIC SAW A. The puparia were not recovered throughout the length of the gutter in CVIC SAW C and the distribution pattern appears to be positively skewed, although within the overall distribution there does appear to still be an oscillating pattern occurring. The ratios of variance to the mean (Table 3.11) conducted on the results from each experimental run produced a ratio much greater than one, suggesting that the distribution of puparia recovered was highly clumped. These results may provide evidence of aggregation prior to pupariation. These results are examined in more detail in the following Sections (Sections 3.3.1.1 and 3.3.1.2) and discussed at the end of this Chapter (Section 3.4).



Figures 3.11.a-c: The percentage of puparia recovered from each 10 cm section (60 sections in total) from each experiment conducted in the 6 m gutter. 'PTER' = *Protophormia terraenovae* and 'SAW' = sawdust substrate. The number of individuals used in each experiment is shown on each graph by 'n'.

Table 3.12: Summary of the ratios of variance to the mean conducted on each experimental run, where a score > 1.0 indicates aggregation and a score closer to 1 suggests a Poisson distribution. 'PTER' = *Protophormia terraenovae* and 'SAW' = sawdust substrate.

	Number of post-feeding larvae	Ratio of variance to the mean
PTER SAW A	263	4.82
PTER SAW B	120	13.02
PTER SAW C	500	67.20

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The results of PTER SAW A appear very similar to those of CVIC WLG A-C and CVIC SAW A and B; puparia were recovered throughout the length of the gutter and in an oscillating pattern. The distribution of the puparia recovered from PTER SAW B and C are different to all of the results reported in this Section. In both instances there appears to have been limited larval dispersal, with all of the puparia recovered within the first 2 m of the gutter. The ratios of variance to the mean (Table 3.12) conducted on the results from each experimental run produced a ratio much greater than one, suggesting that the distribution of puparia recovered was highly clumped. These results may provide evidence of aggregation prior to pupariation. These results are examined in more detail in the following Sections (Sections 3.3.1.1 and 3.3.1.2) and discussed at the end of this Chapter (Section 3.4).

Visual inspection of all of the *C. vicina* results (CVIC WLG A-C, CVIC BAR A-C and CVIC SAW A-C) suggests that there is little difference in the general distribution of the recovered puparia or in the overall distance dispersed by the post-feeding larvae, based on the number of larvae introduced. This is especially clear when comparing CVIC SAW A and CVIC SAW B as n varied from 100 to 1200 and yet the results are similar. Therefore, it was decided to continue to employ a variable n, i.e. the number of larvae reared at the time of the experimental run.

The first set of hypotheses examined the distribution pattern of the puparia recovered from each 10 cm section of the gutter. Ratios of variance to the mean tests were carried out in RStudio® to determine whether the distribution of the number of puparia recovered from each section was uniform. This was done for all 12 experiments and the results of these tests are summarised in Tables 3.9 – 3.12. The results from each test produced a ratio much greater than one, suggesting that the distribution of puparia recovered was highly clumped, so the null hypothesis can be rejected for all the experiments. Visual inspection of the data in each of the figures (Figures 3.8.a-c, 3.9.a-c and 3.10.a-c and 3.11.a-c) clearly shows that the number of puparia recovered in each

sampled section is not normally distributed, so the primary alternative hypothesis can also be rejected for each experiment. In theory, the results of these experiments should allow the acceptance of the secondary alternative hypothesis, a random distribution was most likely occurring during every experiment. However, the patterns of the distribution of puparia throughout all experimental runs (except CVIC BAR A-C, where puparia were concentrated at either end of the gutter) appears to be oscillating, not random. Due to the oscillation pattern occurring almost ubiquitously throughout these results, the author proposes that larval aggregation prior to pupariation may be occurring. This theory is discussed in more depth in Section 3.4.

3.3.1.1 Six metre horizontal dispersal experiment: substrate

The second set of hypotheses examined the differences in dispersal using different substrates for each experiment. A visual inspection of all of the *C. vicina* figures (Figures 3.8.a-c, 3.8.a-c and 3.10.a-c) shows a very different pattern of dispersal between the bare substrate and the other two dispersal substrates tested. The results of the bare substrate show peaks of puparia recovered from either end of the gutter and few puparia recovered from the middle sections; moreover, for each of the experiments over 50 % of the puparia were recovered from the first and last 50 cm of the gutter. This difference is highlighted by the higher percentage of puparia recovered from the last 50 cm of the gutter in the bare substrate, when compared with the other substrates (Table 3.13).

Table 3.13: Comparison of the proportion of *Calliphora vicina* pupariating in the first and last 50 cm of the 6 m long gutter depending on the substrate type ('WLG' = Wildlife Garden soil, 'SAW' = sawdust and 'BAR' = bare (no substrate).

Substrate	**Experiment**	**% puparia recovered from the first 50 cm**	**% puparia recovered from the last 50 cm**
WLG	A	11	9
WLG	B	11	1
WLG	C	40	0
SAW	A	2	9
SAW	B	6	3
SAW	C	43	0
BAR	A	33	24
BAR	B	31	20
BAR	C	45	10

With regards to the hypotheses laid out earlier in this Chapter (Section 3.1.1.1), the results show that H_0 can be rejected and H_1 accepted, as there is clearly a difference in the distribution patterns shown by the puparia recovered from the bare gutter when compared to the Wildlife Garden soil and sawdust substrates. There is also a difference in the overall distance dispersed in each substrate, where the furthest distance dispersed is seen on the bare, plastic substrate.

3.3.1.2 Six metre horizontal dispersal experiment: species

The results of each ratio of variance to the mean test conducted on the results of the *C. vicina* and *P. terraenovae* in sawdust experimental runs are highly significant, showing the experimental runs for both species are not evenly distributed. However, upon further examination, clear differences can be seen in the overall distance dispersed by each species.

The results of the overall distance dispersed by *P. terraenovae* in experimental runs PTER SAW B and C are very different than those of *C. vicina* (CVIC SAW A-C) in the sawdust substrate. Almost 100 % of puparia were recovered from the first 50 cm in PTER B and over 60 % in PTER SAW C, compared to less than 10 % of puparia in CVIC SAW A and B and less than 45 % of puparia in CVIC SAW C. Moreover, over 60 % of *P. terraenovae* puparia were recovered from the first 10 cm of the gutter in PTER SAW B. This information is summarised in Table 3.14. The results of PTER SAW B and C suggest that *P. terraenovae* disperses horizontally much less than *C. vicina*. The results of PTER SAW A are interesting as they more closely mimic those of the *C. vicina* runs, the reason for the difference of the results for the PTER runs is unknown as, to the author's knowledge, no biotic or abiotic factors were different. These results highlight the complexity of horizontal dispersal and demonstrate that there are unknown factors that are involved that could be intrinsic to *P. terraenovae* or perhaps there may be some factors that affect horizontal dispersal that are currently unknown.

Table 3.14: Comparison of the proportion of *Calliphora vicina* and *Protophormia terraenovae* pupariating in the first 50 cm and 10 cm of the 6 m long gutter in sawdust substrate.

Species	Experiment	% puparia recovered from the first 50 cm	% puparia recovered from the first 10 cm
CVIC	A	2	0
CVIC	B	6	0
CVIC	C	43	10
PTER	A	15	7
PTER	B	97	62
PTER	C	63	13

3.3.2 One metre horizontal dispersal experiment

This Section consists of 56 experimental runs conducted using *C. vicina, L. sericata, C. vomitoria* and *P. terraenovae* post-feeding larvae. Four different dispersal substrates were used: Wildlife Garden topsoil, sawdust, commercial soil and no substrate (bare, plastic gutter). These experiments were conducted to examine any differences in the distribution pattern of the puparia recovered, depending on the substrate and/or species.

In order to examine the distribution of the puparia recovered from each section further, the frequency of puparia recovered was plotted and ratios of variance to the mean tests were carried out in RStudio®. The results of these experimental runs are shown:

- CVIC WLG Figures 3.12.a-g, ratio of variance to the mean Table 3.15 and frequency graph 3.13.
- CVIC SAW Figures 3.14.a-l, ratio of variance to the mean Table 3.16 and frequency graph 3.15.
- CVIC BAR Figures 3.16.a-l, ratio of variance to the mean Table 3.17 and frequency graph 3.17.
- CVIC COM Figures 3.18.a-e, ratio of variance to the mean Table 3.18 and frequency graph 3.18.
- LSER BAR Figures 3.20.1-e, ratio of variance to the mean Table 3.19and frequency graph 3.20.
- LSER COM Figures 3.22.a-e, ratio of variance to the mean Table 3.20 and frequency graph 3.22.
- CVOM COM Figures 3.24.a-e, ratio of variance to the mean Table 3.21 and frequency graph 3.24.
- PTER SAW Figures 3.26.a-f, ratio of variance to the mean Table 3.22 and frequency graph 3.26.

In all of the experimental runs set out in this Section, the post-feeding larvae were introduced to the middle of the gutter (50 cm).

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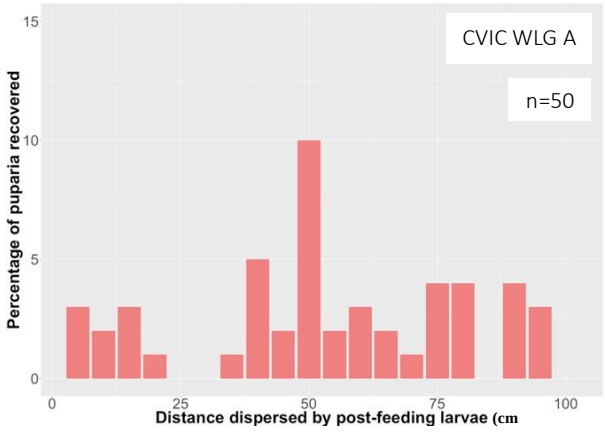
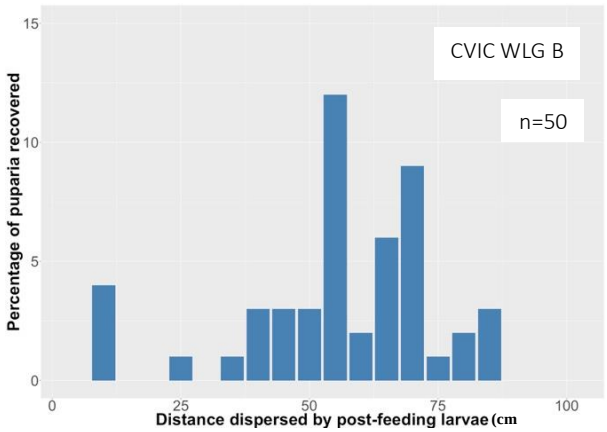
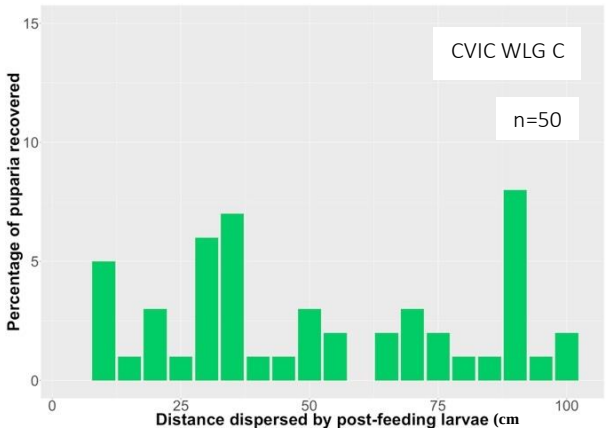
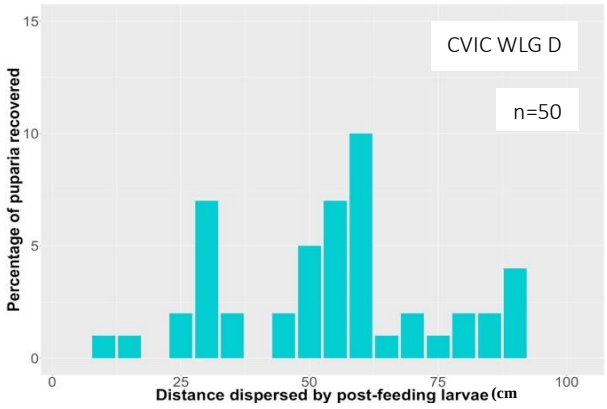
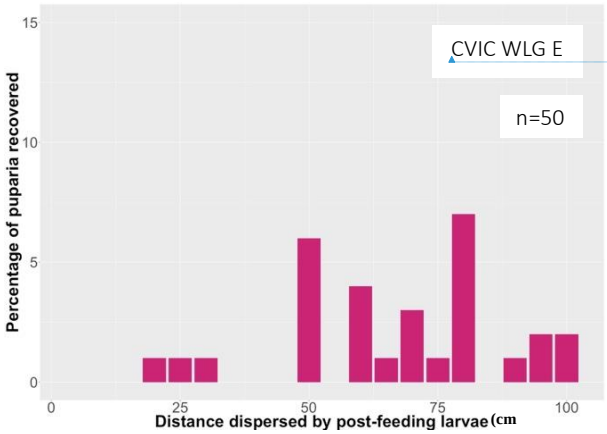


Figures 3.12.a-g: The percentage of puparia recovered from each 5 cm section (20 sections in total) from each experiment conducted in the 1 m gutter. 'CVIC' = *Calliphora vicina* and 'WLG' = Wildlife Garden soil substrate. The number of individuals used in each experiment is shown on each graph by 'n'.


CVIC WLG B
n=50
Percentage of puparia recovered
Distance dispersed by post-feeding larvae (cm


CVIC WLG C
n=50
Percentage of puparia recovered
Distance dispersed by post-feeding larvae (cm


CVIC WLG D
n=50
Percentage of puparia recovered
Distance dispersed by post-feeding larvae (cm



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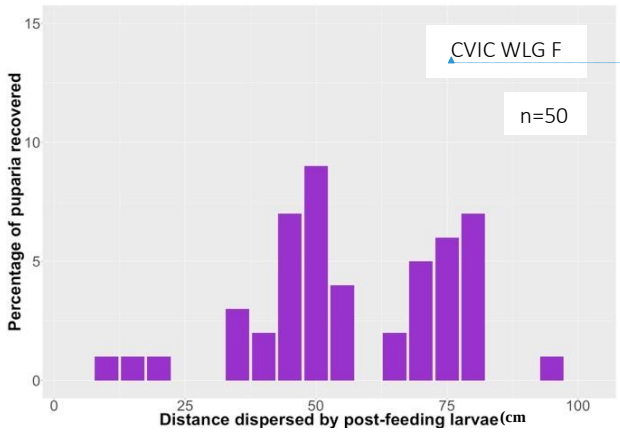

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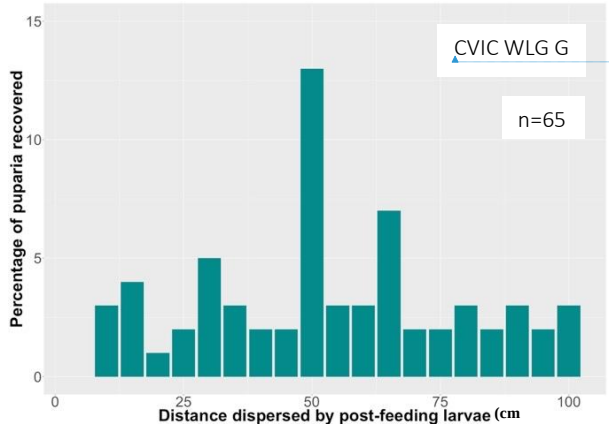

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Table 3.15: Summary of the ratios of variance to the mean conducted on each experimental run, where a score > 1.0 indicates aggregation and a score closer to 1 suggests a Poisson distribution. 'CVIC' = *Calliphora vicina* and 'WLG' = Wildlife Garden soil substrate.

	Number of post-feeding larvae	Ratio of variance to the mean
CVIC WLG A	50	2.17
CVIC WLG B	50	4.19
CVIC WLG C	50	2.08
CVIC WLG D	50	3.16
CVIC WLG E	50	6.27
CVIC WLG F	50	3.371
CVIC WLG G	65	2.26

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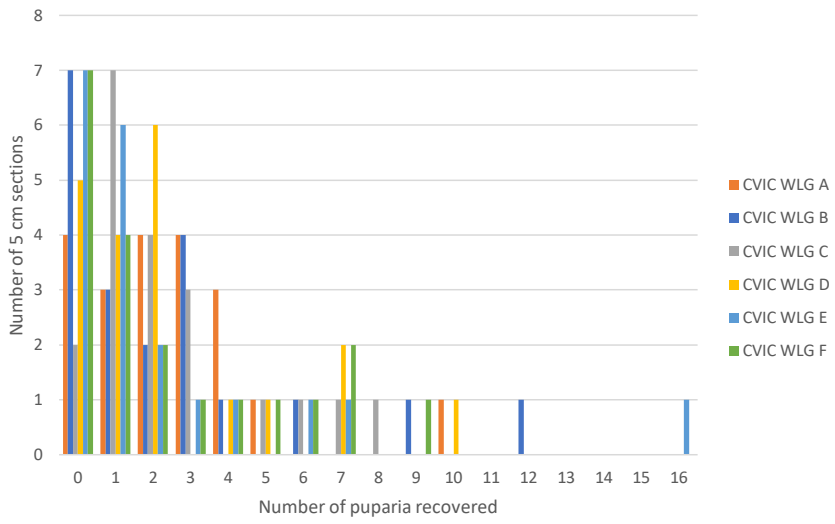
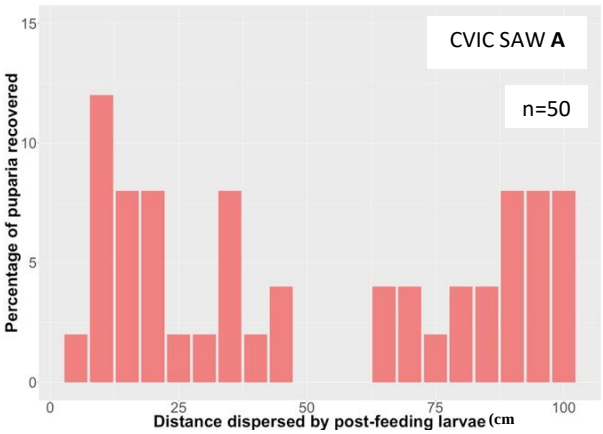
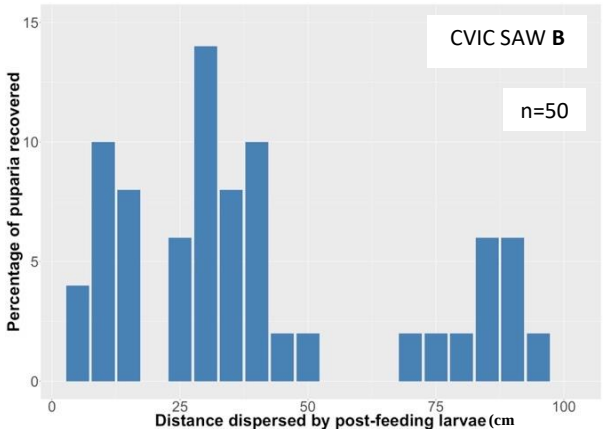
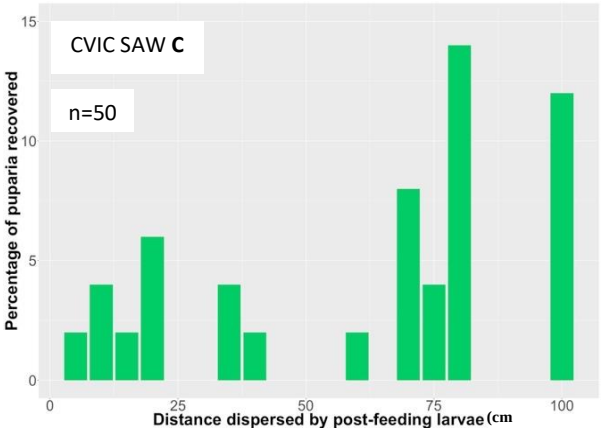


Figure 3.13: The number of 5 cm sections that each number of puparia were recovered from in each experimental run. 'CVIC' = *Calliphora vicina* and 'WLG' = Wildlife Garden soil substrate. n = 50 (CVIC WLG G is excluded from this graph as n = 65 and therefore was not a fair comparison).

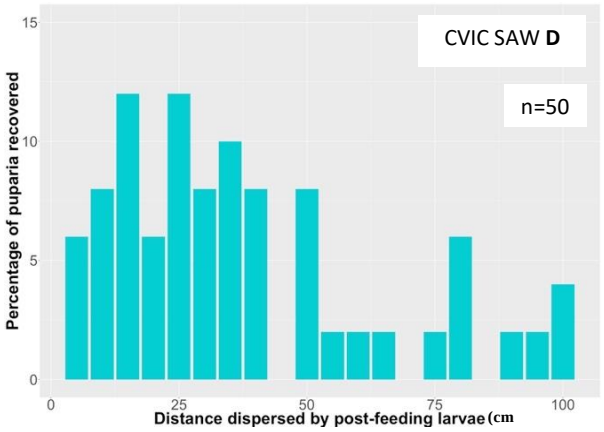
The results of CVIC WLG A-G appear similar. The puparia were recovered throughout the length of the gutter in each case and the distribution of puparia appears to be an oscillating pattern. The ratios of variance to the mean (Table 3.15) conducted on the results from each experimental run produced a ratio much greater than one, suggesting that the distribution of puparia recovered was highly clumped. Moreover, the frequency graph (Figure 3.13) shows that the number of puparia recovered from each 5 cm section of the gutter was not consistent throughout the experimental runs. All of the analyses may provide further evidence of aggregation prior to pupariation. The results are discussed in further detail later in this Section.

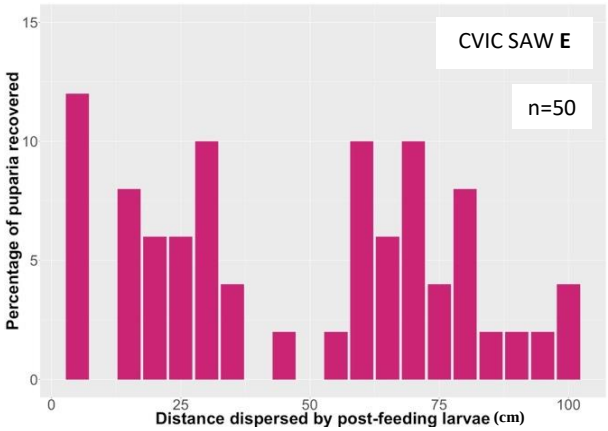


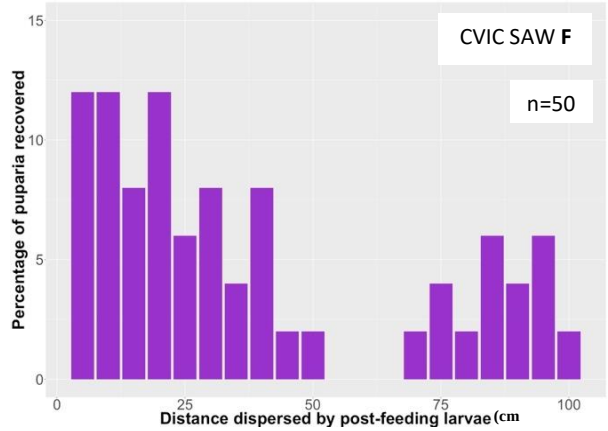


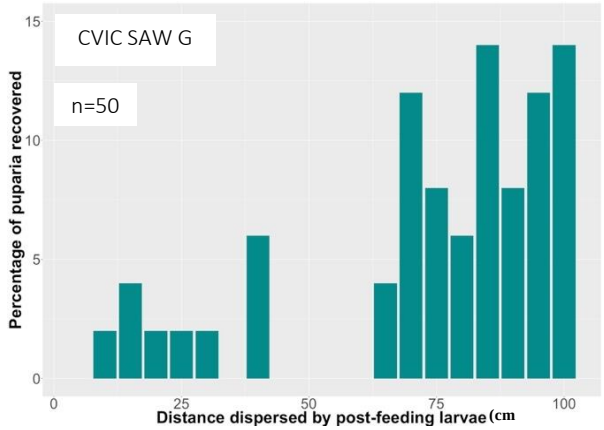


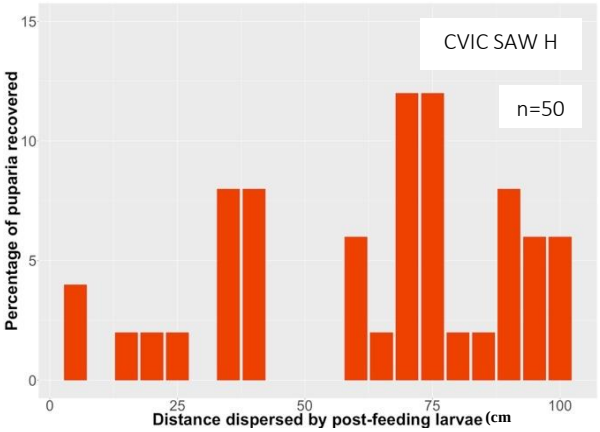
Figures 3.14.a-l: The percentage of puparia recovered from each 5 cm section (20 sections in total) from each experiment conducted in the 1 m gutter. 'CVIC' = *Calliphora vicina* and 'SAW' = sawdust substrate. The number of individuals used in each experiment is shown on each graph by 'n'.

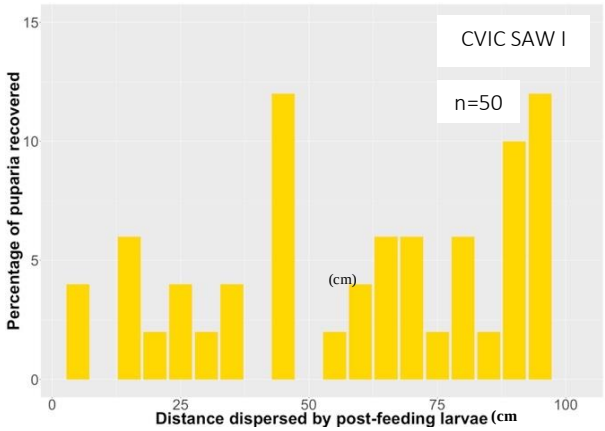

CVIC SAW D
n=50
Percentage of puparia recovered
Distance dispersed by post-feeding larvae (cm

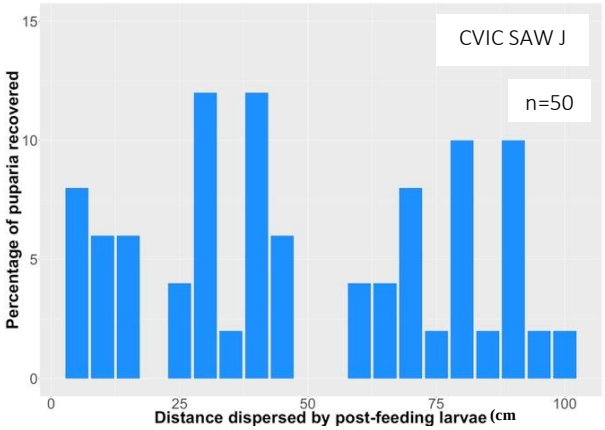

CVIC SAW E
n=50
Percentage of puparia recovered
Distance dispersed by post-feeding larvae (cm)

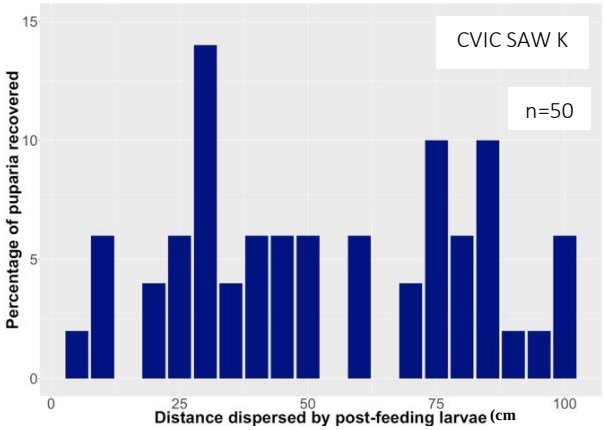

CVIC SAW F
n=50
Percentage of puparia recovered
Distance dispersed by post-feeding larvae (cm


CVIC SAW G
n=50
Percentage of puparia recovered
Distance dispersed by post-feeding larvae (cm


CVIC SAW H
n=50
Percentage of puparia recovered
Distance dispersed by post-feeding larvae (cm


CVIC SAW I
n=50
Percentage of puparia recovered
(cm)
Distance dispersed by post-feeding larvae (cm


CVIC SAW J
n=50
Percentage of puparia recovered
Distance dispersed by post-feeding larvae (cm


CVIC SAW K
n=50
Percentage of puparia recovered
Distance dispersed by post-feeding larvae (cm

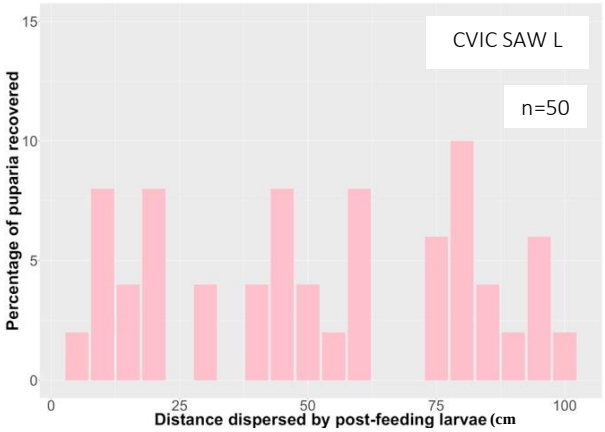

CVIC SAW L
n=50
Percentage of puparia recovered
Distance dispersed by post-feeding larvae (cm

Table 3.16: Summary of the ratios of variance to the mean conducted on each experimental run, where a score > 1.0 indicates aggregation and a score closer to 1 suggests a Poisson distribution. 'CVIC' = *Calliphora vicina* and 'SAW' = sawdust substrate.

	Number of post-feeding larvae	Ratio of variance to the mean
CVIC SAW **A**	50	2.61
CVIC SAW **B**	50	4.51
CVIC SAW **C**	50	7.67
CVIC SAW **D**	50	3.07
CVIC SAW **E**	50	2.88
CVIC SAW **F**	50	3.16
CVIC SAW **G**	50	5.12
CVIC SAW **H**	50	4.34
CVIC SAW **I**	50	3.92
CVIC SAW **J**	50	3.07
CVIC SAW **K**	50	2.57
CVIC SAW **L**	50	3.66

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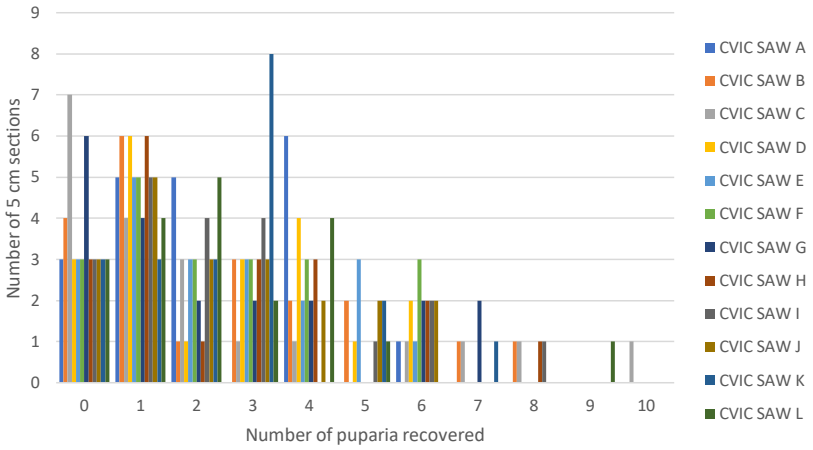
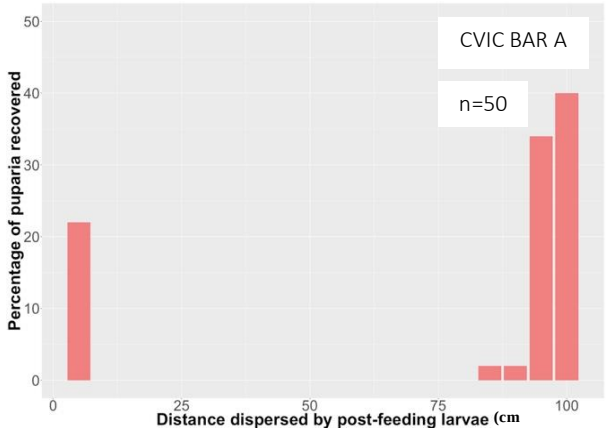
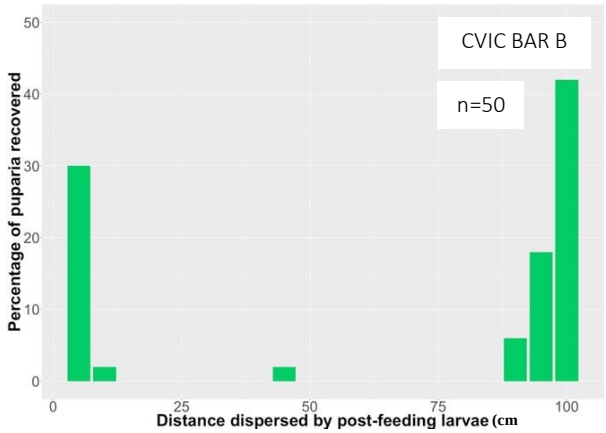


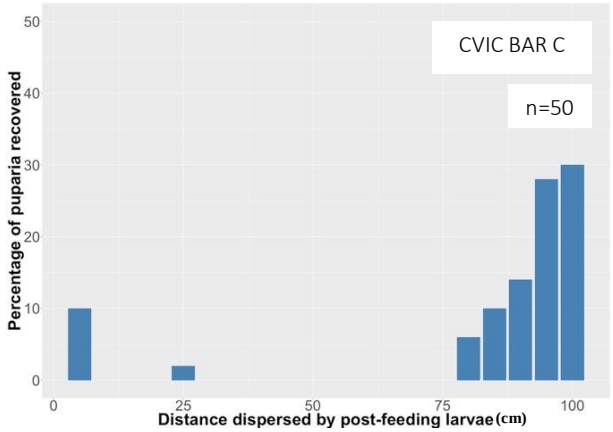
Figure 3.15: The number of 5 cm sections that each number of puparia were recovered from in each experimental run. 'CVIC' = *Calliphora vicina* and 'SAW' = sawdust substrate. n = 50.

The results of CVIC SAW A-L appear similar to each other. Again, the puparia were recovered throughout the length of the gutter in each case and the distribution of puparia appears to be an oscillating pattern. There appears to be a slight tendency, in the results of each run, for the

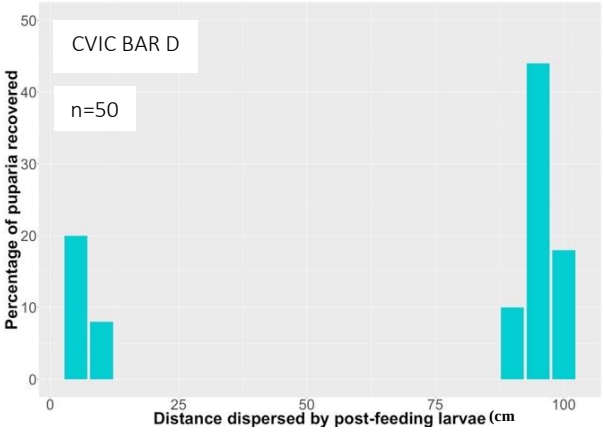
puparia to be recovered from either end, such that there is a slight gap in the pattern that corresponds to the middle of the gutter, where no puparia were located. The location of the gap varied between runs, but was always in the middle third of the gutter, apart from in CVIC SAW L, where no puparia were recovered between 65 and 70 cm from the start of the gutter. The ratios of variance to the mean (Table 3.16) conducted on the results from each experimental run produced a ratio much greater than one, suggesting that the distribution of puparia recovered was highly clumped. Moreover, the frequency graph (Figure 3.15) shows that the number of puparia recovered from each 5 cm section of the gutter was not consistent throughout the experimental runs. All of the analyses may provide further evidence of aggregation prior to pupariation. The results are discussed in further detail later in this Section.

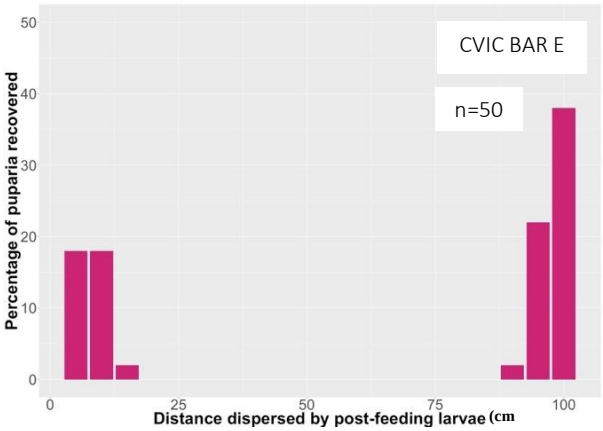


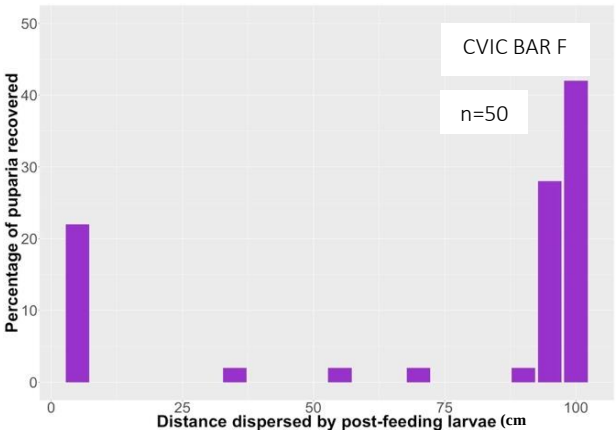


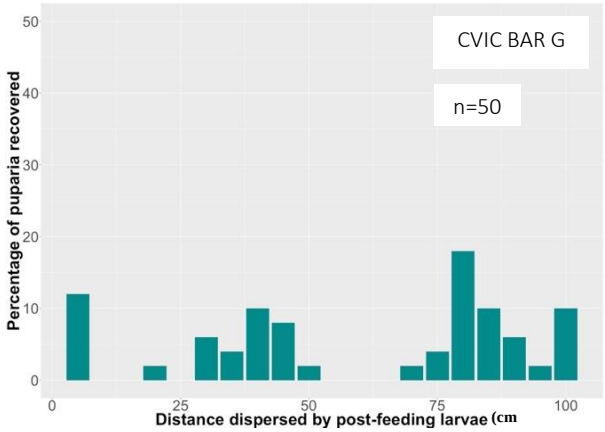


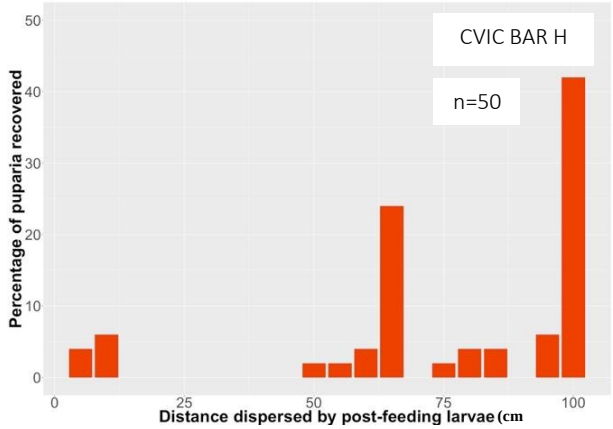
Figures 3.16.a-k: The percentage of puparia recovered from each 5 cm section (20 sections in total) from each experiment conducted in the 1 m gutter. 'CVIC' = *Calliphora vicina* and 'BAR' = bare (no substrate). The number of individuals used in each experiment is shown on each graph by 'n'.

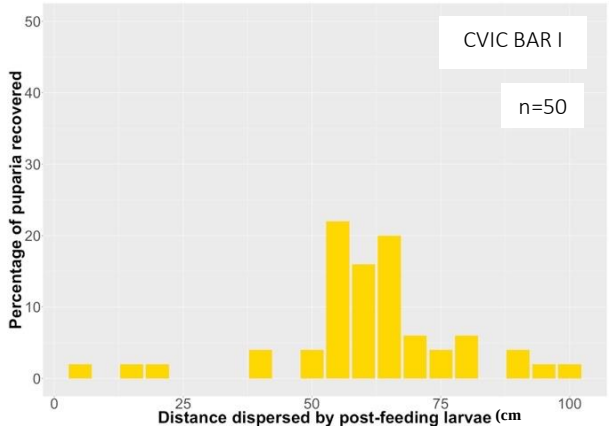

CVIC BAR D
n=50
Percentage of puparia recovered
0
10
20
30
40
50
0
25
50
75
100
Distance dispersed by post-feeding larvae (cm

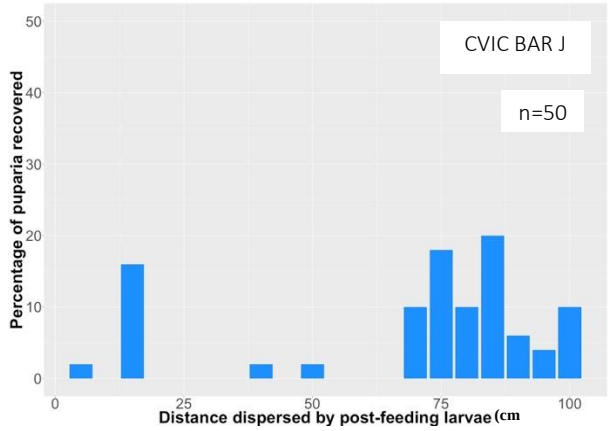

CVIC BAR E
n=50
Percentage of puparia recovered
0
10
20
30
40
50
0
25
50
75
100
Distance dispersed by post-feeding larvae (cm


CVIC BAR F
n=50
Percentage of puparia recovered
0
10
20
30
40
50
0
25
50
75
100
Distance dispersed by post-feeding larvae (cm


CVIC BAR G
n=50
Percentage of puparia recovered
0
10
20
30
40
50
0
25
50
75
100
Distance dispersed by post-feeding larvae (cm


CVIC BAR H
n=50
Percentage of puparia recovered
0
10
20
30
40
50
0
25
50
75
100
Distance dispersed by post-feeding larvae (cm


CVIC BAR I
n=50
Percentage of puparia recovered
0
10
20
30
40
50
0
25
50
75
100
Distance dispersed by post-feeding larvae (cm


CVIC BAR J
n=50
Percentage of puparia recovered
Distance dispersed by post-feeding larvae (cm

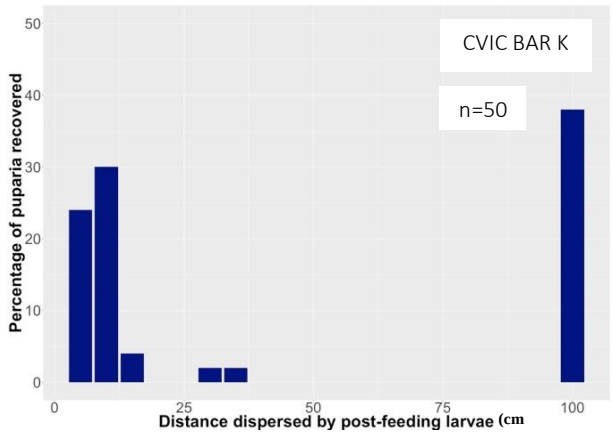

CVIC BAR K
n=50
Percentage of puparia recovered
Distance dispersed by post-feeding larvae (cm

Table 3.17: Summary of the ratios of variance to the mean conducted on each experimental run, where a score > 1.0 indicates aggregation and a score closer to 1 suggests a Poisson distribution. 'CVIC' = *Calliphora vicina* and 'BAR' = bare (no substrate).

	Number of post-feeding larvae	Ratio of variance to the mean
CVIC BAR **A**	50	28.93
CVIC BAR **B**	50	17.05
CVIC BAR **C**	50	26.65
CVIC BAR **D**	50	24.46
CVIC BAR **E**	50	21.94
CVIC BAR **F**	50	26.82
CVIC BAR **G**	50	5.39
CVIC BAR **H**	50	20.93
CVIC BAR **I**	50	9.16
CVIC BAR **J**	50	10.09
CVIC BAR **K**	50	25.73

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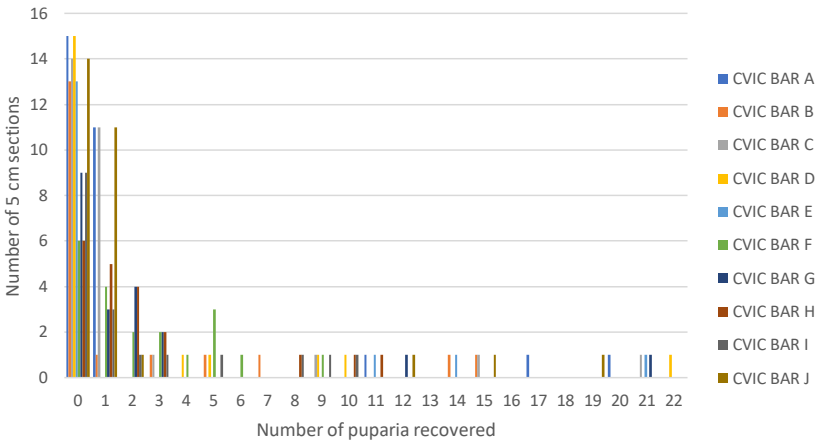
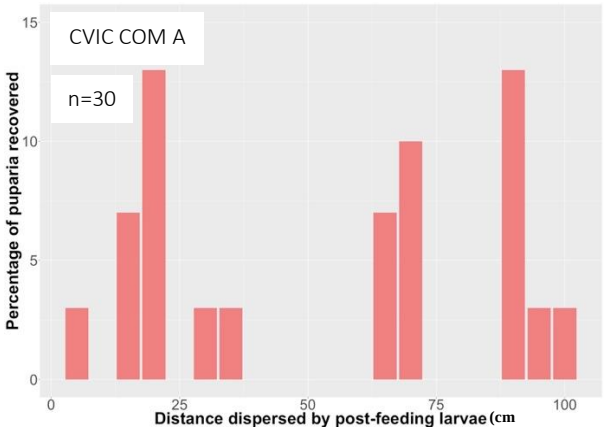
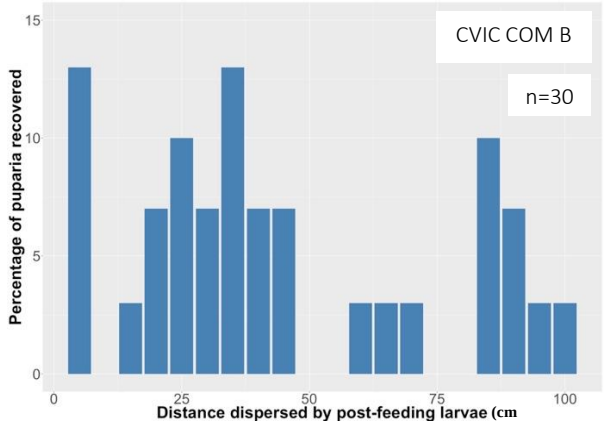


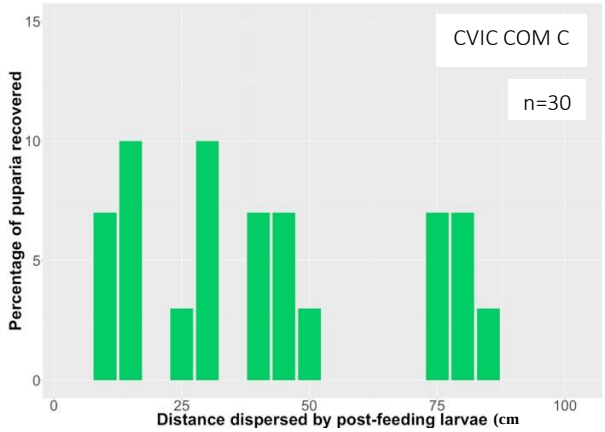
Figure 3.17: The number of 5 cm sections that each number of puparia were recovered from in each experimental run. 'CVIC' = *Calliphora vicina* and 'BAR' = bare (no substrate). n = 50.

The results of CVIC BAR A-K appear similar to each other. The majority of puparia were recovered from either end of the gutters, similar to the results of *C. vicina* in the bare 6 m gutter (Section

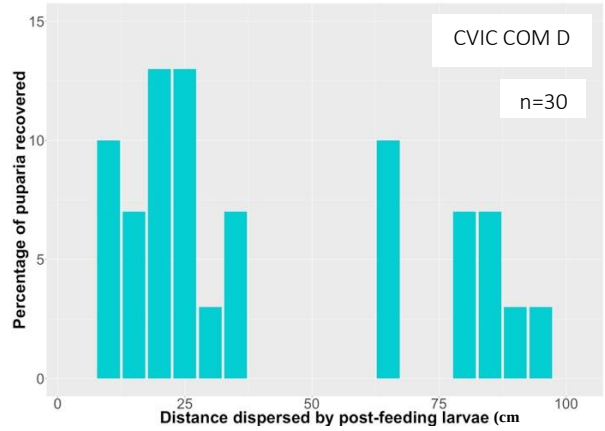
3.3.1). Again, the frequency graph (Figure 3.17) shows that the number of puparia recovered from each 5 cm section of the gutter was not consistent throughout the experimental runs. Moreover, Figure 3.17 shows greater numbers of puparia recovered in comparison with the frequency graphs of the other experimental runs. Again, the ratios of variance to the mean (Table 3.17) conducted on the results from each experimental run produced a ratio much greater than one, suggesting that the distribution of puparia recovered was highly clumped. The distribution patterns produced in these experimental runs are very different to those of the other experimental runs reported in this Section (the results are similar to LSER BAR A-E reported later in this Section) and are likely due to the lack of substrate used (bare gutter). The results are discussed in further detail later in this Section.







Figures 3.18.a-e: The percentage of puparia recovered from each 5 cm section (20 sections in total) from each experiment conducted in the 1 m gutter. 'CVIC' = *Calliphora vicina* and 'COM' = commercial topsoil substrate. The number of individuals used in each experiment is shown on each graph by 'n'.



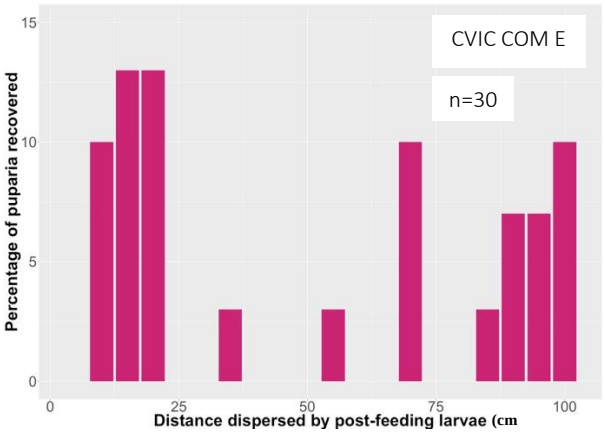


Table 3.18: Summary of the ratios of variance to the mean conducted on each experimental run, where a score > 1.0 indicates aggregation and a score closer to 1 suggests a Poisson distribution. 'CVIC' = *Calliphora vicina* and COM''= commercial topsoil substrate.

	Number of post-feeding larvae	Ratio of variance to the mean
CVIC COM **A**	30	7.11
CVIC COM **B**	30	3.69
CVIC COM **C**	30	6.78
CVIC COM **D**	30	5.79
CVIC COM **E**	30	7.16

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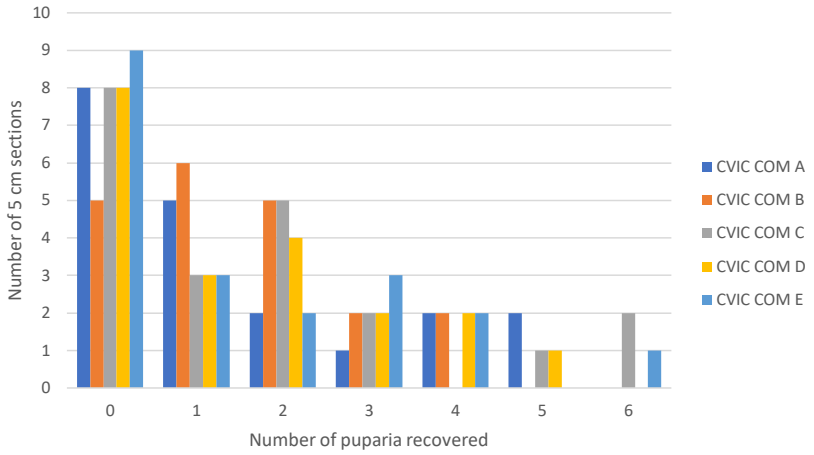
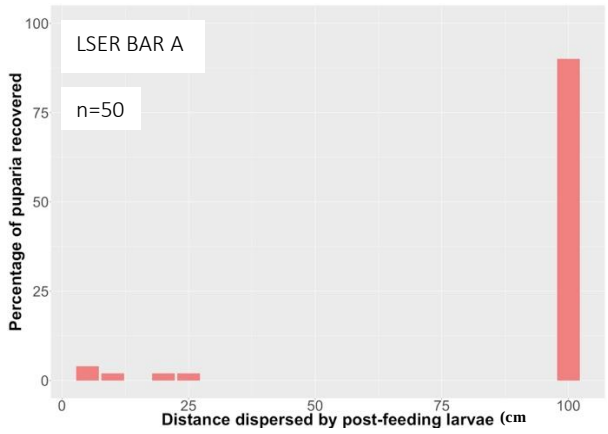
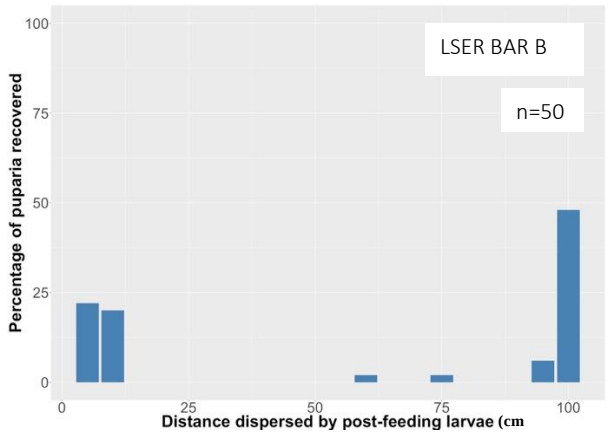
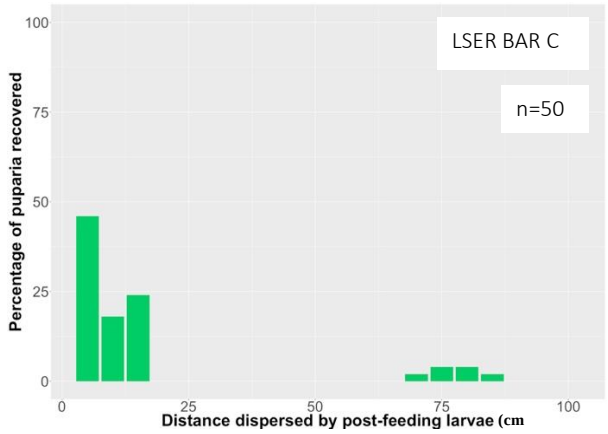


Figure 3.19: The number of 5 cm sections that each number of puparia were recovered from in each experimental run. 'CVIC' = *Calliphora vicina* and 'COM' = commercial topsoil substrate. n = 30.

The results of CVIC COM A-E look similar. The puparia were recovered throughout the length of the gutter in each case and the distribution of puparia appears to be an oscillating pattern. Similar to the results of *C. vicina* in sawdust (CVIC SAW A-L), there appears to be a slight tendency, in the results of each run, for the puparia to be recovered from either end, such that there is a slight gap in the pattern that corresponds to the middle of the gutter, where no puparia were located. Again, the frequency graph (Figure 3.19) shows that the number of puparia recovered from each 5 cm section of the gutter was not consistent throughout the experimental runs, although the range of the number of puparia recovered is much less than the other frequency graphs (Figures 3.13, 3.15 and 3.17), with a maximum of 6 puparia recovered from a 5 cm section. This was expected as the number of larvae used during this set of runs was 30, less than the other runs. Again, the ratios of variance to the mean (Table 3.18) conducted on the results from each experimental run produced a ratio much greater than one, suggesting that the distribution of puparia recovered was highly clumped. All of the analyses may provide further evidence of aggregation prior to pupariation. The results are discussed in further detail later in this Section.







Figures 3.20.a-e: The percentage of puparia recovered from each 5 cm section (20 sections in total) from each experiment conducted in the 1 m gutter. 'LSER' = *Lucilia sericata* and 'BAR' = bare (no substrate). The number of individuals used in each experiment is shown on each graph by 'n'.

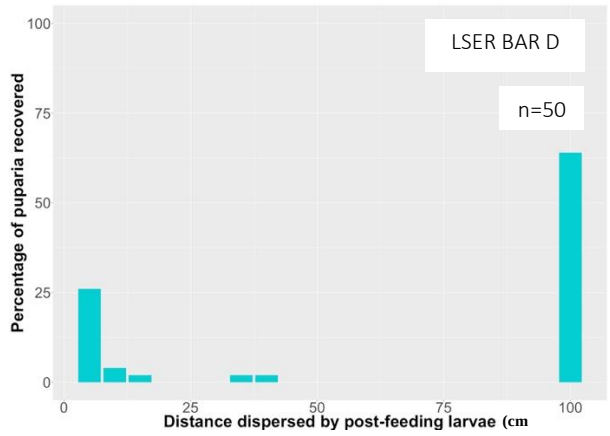


Table 3.19: Summary of the ratios of variance to the mean conducted on each experimental run, where a score > 1.0 indicates aggregation and a score closer to 1 suggests a Poisson distribution. 'LSER' = *Lucilia sericata* and 'BAR' = bare (no substrate).

	Number of post-feeding larvae	Ratio of variance to the mean
LSER BAR **A**	50	80.29
LSER BAR **B**	50	8.76
LSER BAR **C**	50	26.91
LSER BAR **D**	50	45.26
LSER BAR **E**	50	26.03

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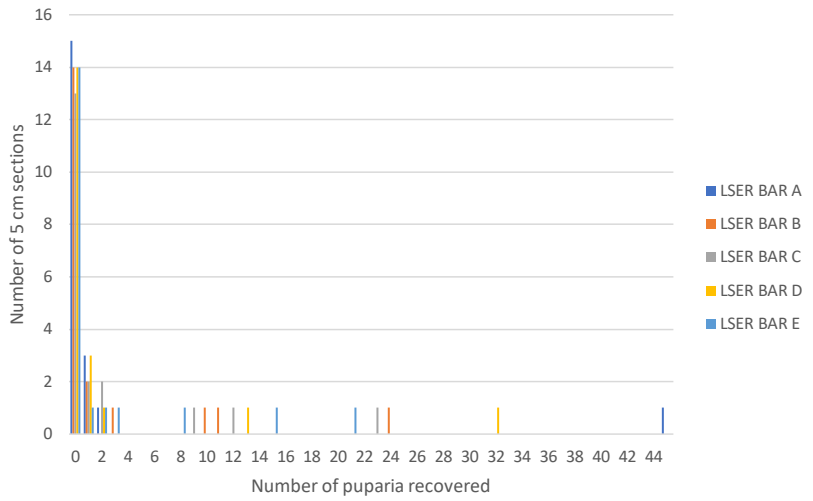
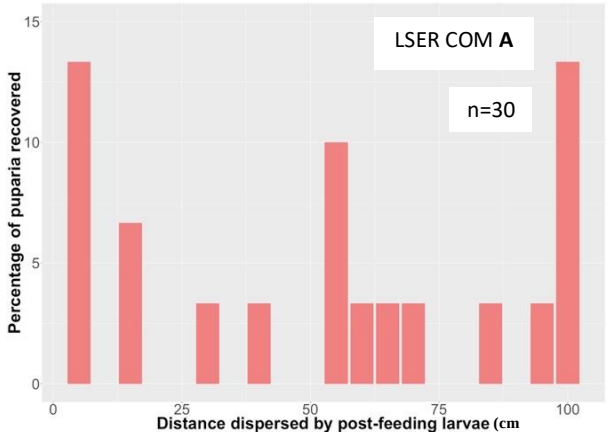
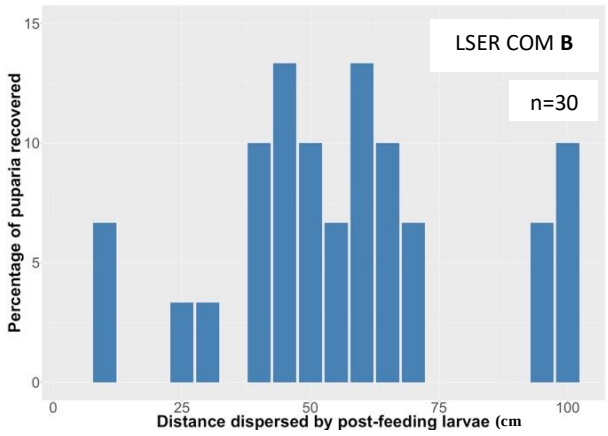
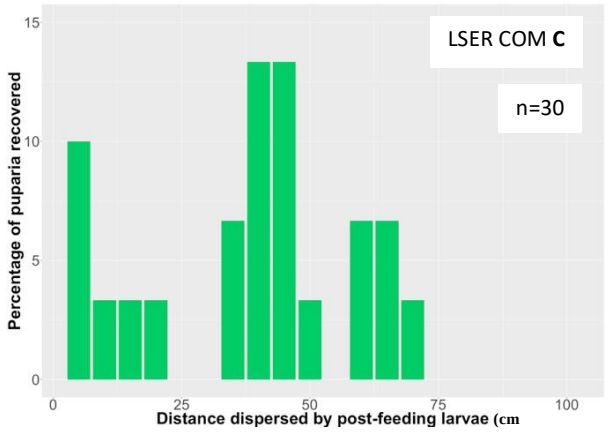


Figure 3.21: The number of 5 cm sections that each number of puparia were recovered from in each experimental run. 'LSER' = *Lucilia sericata* and 'BAR' = bare (no substrate). n = 50.

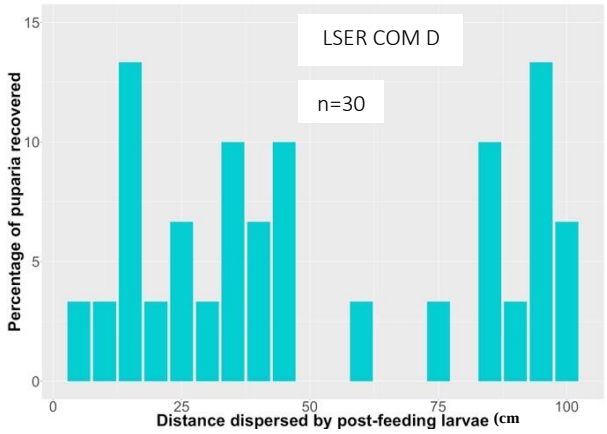
The results of LSER A-E appear similar. The distribution pattern of the puparia recovered was also very similar to that of *C. vicina* in the bare gutter (CVIC BAR A-K); the majority of puparia were recovered from either end of the gutters, again similar to the results of *C. vicina* in the bare 6 m gutter (Section 3.3.1). The frequency graph (Figure 3.21) shows that the number of puparia recovered from each 5 cm section of the gutter was not consistent throughout the experimental runs. Moreover, Figure 3.21 shows greater numbers of puparia recovered in comparison with the frequency graphs of the other experimental runs. Again, the ratios of variance to the mean (Table 3.19) conducted on the results from each experimental run produced a ratio much greater than one, suggesting that the distribution of puparia recovered was highly clumped. The distribution patterns produced in these experimental runs are very different to those of the other experimental runs reported in this Section, apart from CVIC BAR A-K, and are likely due to the lack of substrate used (bare gutter). The results are discussed in further detail later in this Section.







Figures 3.22.a-e: The percentage of puparia recovered from each 5 cm section (20 sections in total) from each experiment conducted in the 1 m gutter. 'LSER' = *Lucilia sericata* and 'COM' = commercial topsoil substrate. The number of individuals used in each experiment is shown on each graph by 'n'.



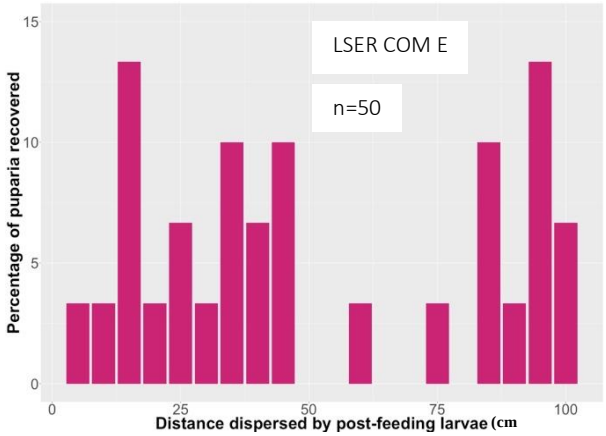


Table 3.20: Summary of the ratios of variance to the mean conducted on each experimental run, where a score > 1.0 indicates aggregation and a score closer to 1 suggests a Poisson distribution. 'LSER' = *Lucilia sericata* and 'COM' = commercial topsoil substrate.

	Number of post-feeding larvae	Ratio of variance to the mean
LSER COM **A**	30	6.67
LSER COM **B**	30	4.79
LSER COM **C**	30	9.01
LSER COM **D**	30	3.81
LSER COM **E**	50	3.81

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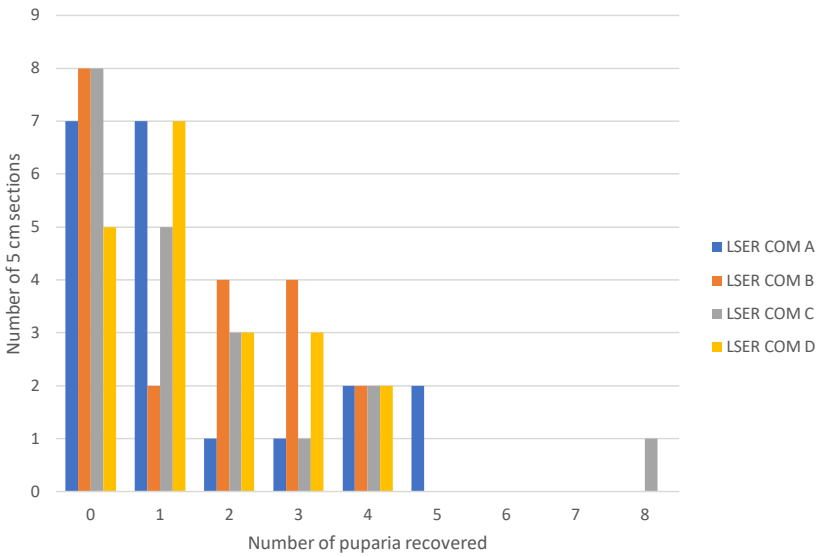
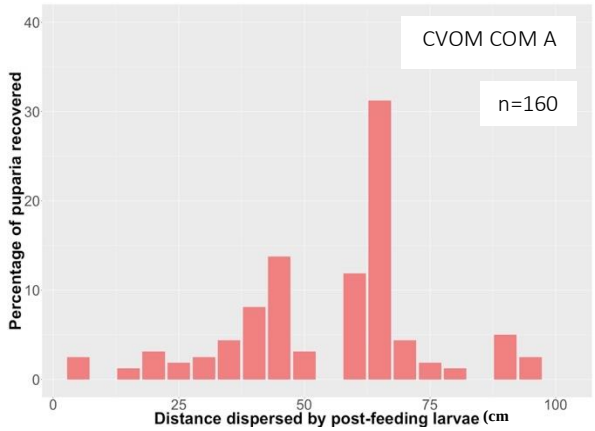
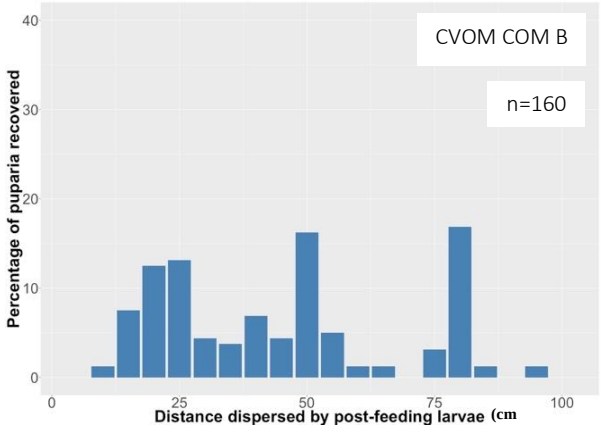
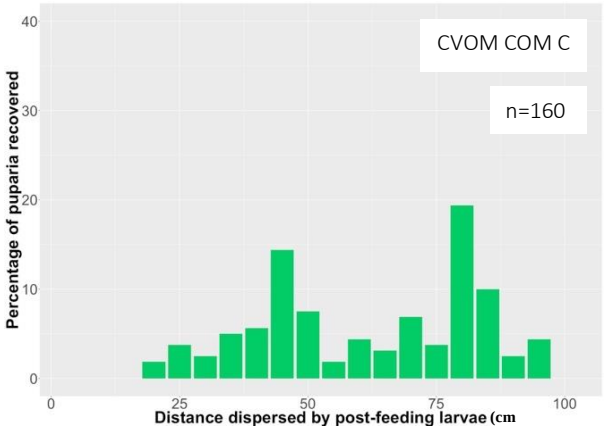


Figure 3.23: The number of 5 cm sections that each number of puparia were recovered from in each experimental run. 'LSER' = *Lucilia sericata* and 'COM' = commercial topsoil substrate. n = 30.

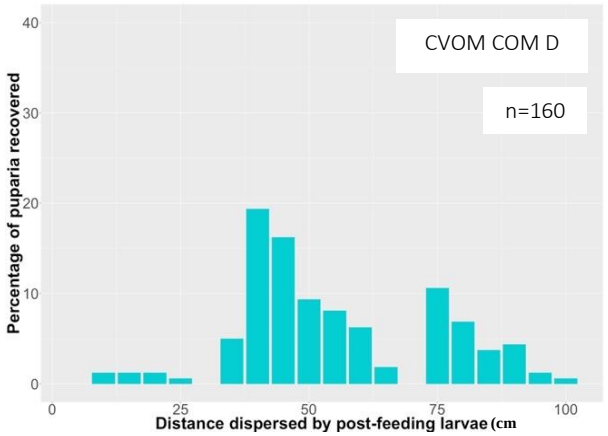
The results of LSER COM A-D look similar. These results appear similar to the results of *C. vicina* in commercial soil (CVIC COM A-E). The puparia were recovered throughout the length of the gutter in each case and the distribution of puparia appears to be an oscillating pattern. Again, the frequency graph (Figure 3.23) shows that the number of puparia recovered from each 5 cm section of the gutter was not consistent throughout the experimental runs. Again, the ratios of variance to the mean (Table 3.20) conducted on the results from each experimental run produced a ratio much greater than one, suggesting that the distribution of puparia recovered was highly clumped. All of the analyses may provide further evidence of aggregation prior to pupariation. The results are discussed in further detail later in this Section.







Figures 3.24.a-e: The percentage of puparia recovered from each 5 cm section (20 sections in total) from each experiment conducted in the 1 m gutter. 'CVOM' = *Calliphora vomitoria* and 'COM' = commercial topsoil substrate. The number of individuals used in each experiment is shown on each graph by 'n'.



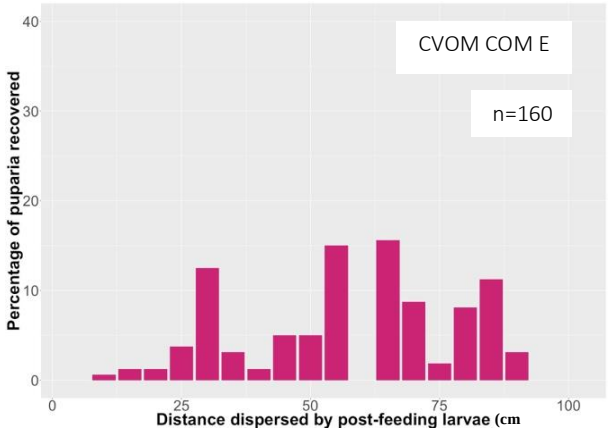


Table 3.21: Summary of the ratios of variance to the mean conducted on each experimental run, where a score > 1.0 indicates aggregation and a score closer to 1 suggests a Poisson distribution. 'CVOM' = *Calliphora vomitoria* and 'COM' = commercial topsoil substrate.

	Number of post-feeding larvae	**Ratio of variance to the mean**
CVOM COM **A**	160	10.49
CVOM COM **B**	160	6.06
CVOM COM **C**	160	4.93
CVOM COM **D**	160	6.10
CVOM COM **E**	160	5.38

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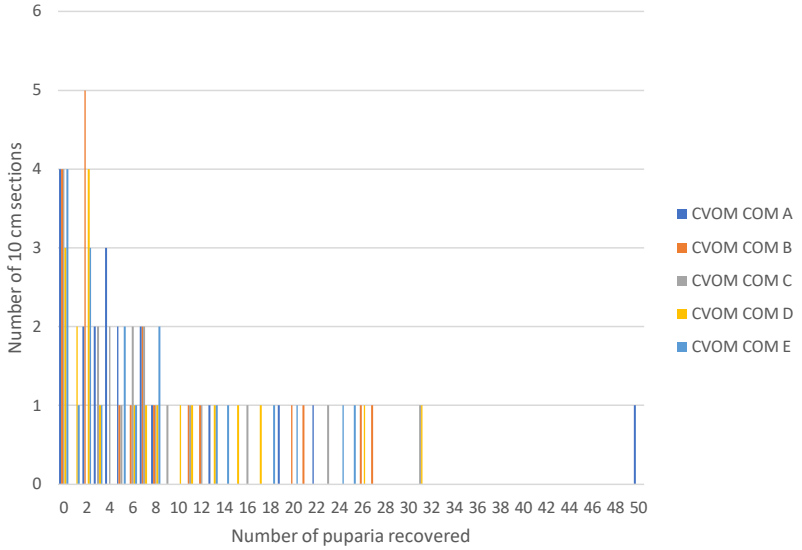
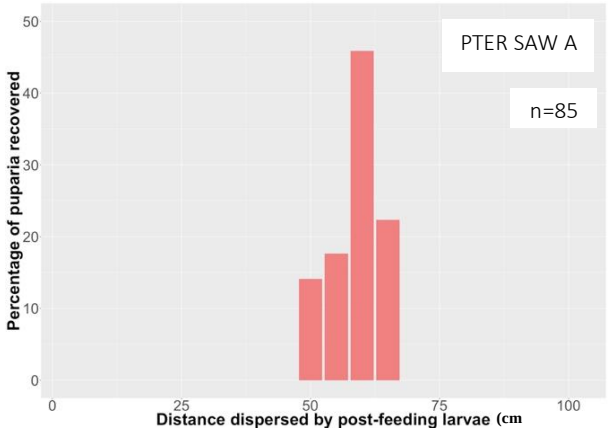
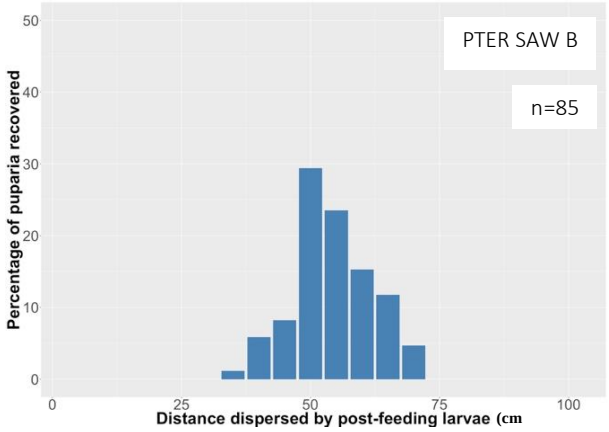
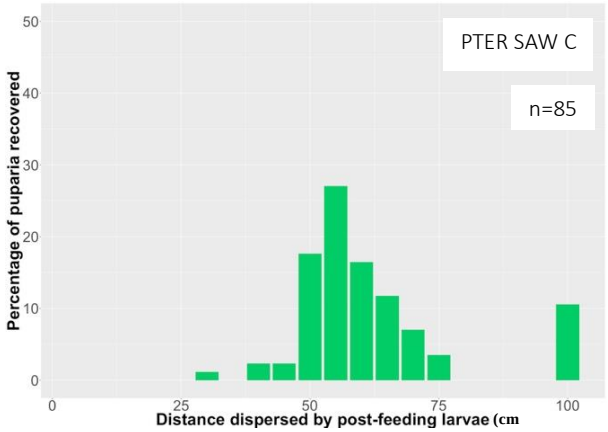


Figure 3.25: The number of 5 cm sections that each number of puparia were recovered from in each experimental run. 'CVOM' = *Calliphora vomitoria* and 'COM' = commercial topsoil substrate. n = 160.

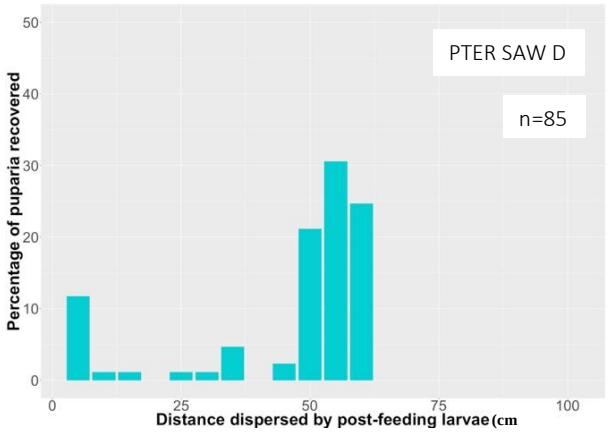
The results of CVOM COM A-E look similar. The results appear similar to those of *C. vicina* and *L. sericata* in commercial soil (CVIC A-E and LSER A-D). The puparia were recovered throughout the length of the gutter in each case and the distribution of puparia appears to be an oscillating pattern. Again, the frequency graph (Figure 3.25) shows that the number of puparia recovered from each 5 cm section of the gutter was not consistent throughout the experimental runs, although the range of the number of puparia recovered is much greater that the other frequency graphs for commercial soil (Figures 3.18, 3.22 and 3.24), with a maximum of 50 puparia recovered from a 5 cm section. This however, may be due to the higher n value of these experiments: 160, compared to 30 and 50. Again, the ratios of variance to the mean (Table 3.21) conducted on the results from each experimental run produced a ratio much greater than one, suggesting that the distribution of puparia recovered was highly clumped. All of the analyses may provide further evidence of aggregation prior to pupariation. The results are discussed in further detail later in this Section.

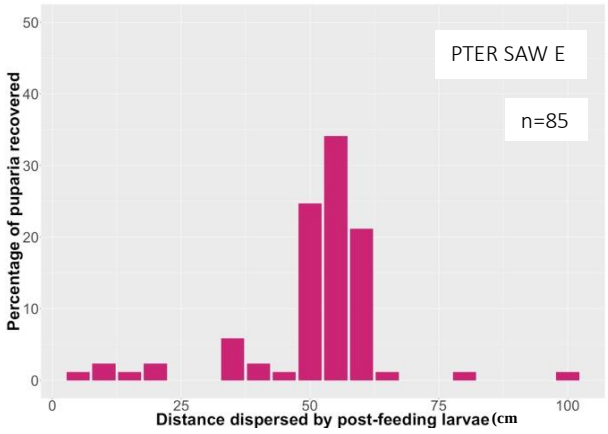






Figures 3.26.a-f: The percentage of puparia recovered from each 5 cm section (20 sections in total) from each experiment conducted in the 1 m gutter. 'PTER' = *Protophormia terraenovae* and 'SAW' = sawdust substrate. The number of individuals used in each experiment is shown on each graph by 'n'.


PTER SAW D
n=85
Percentage of puparia recovered
Distance dispersed by post-feeding larvae (cm


PTER SAW E
n=85
Percentage of puparia recovered
Distance dispersed by post-feeding larvae (cm

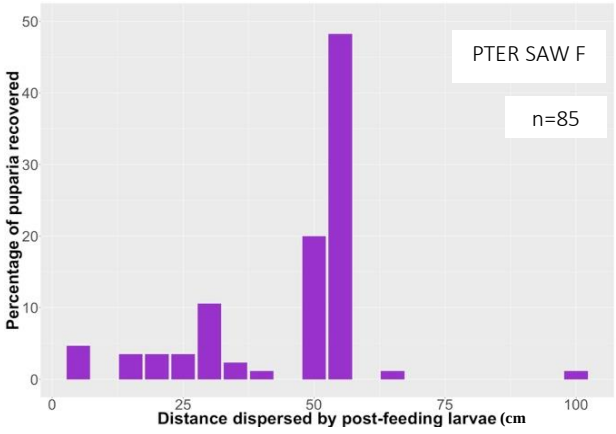

PTER SAW F
n=85
Percentage of puparia recovered
Distance dispersed by post-feeding larvae (cm

Table 3.22: Summary of the ratios of variance to the mean conducted on each experimental run, where a score > 1.0 indicates aggregation and a score closer to 1 suggests a Poisson distribution. 'PTER' = *Protophormia terraenovae* and 'SAW' = sawdust substrate.

	N	Ratio of variance to the mean
PTER SAW **A**	85	16.67
PTER SAW **B**	85	14.92
PTER SAW **C**	85	12.00
PTER SAW **D**	85	17.54
PTER SAW **E**	85	18.76
PTER SAW **F**	85	25.35

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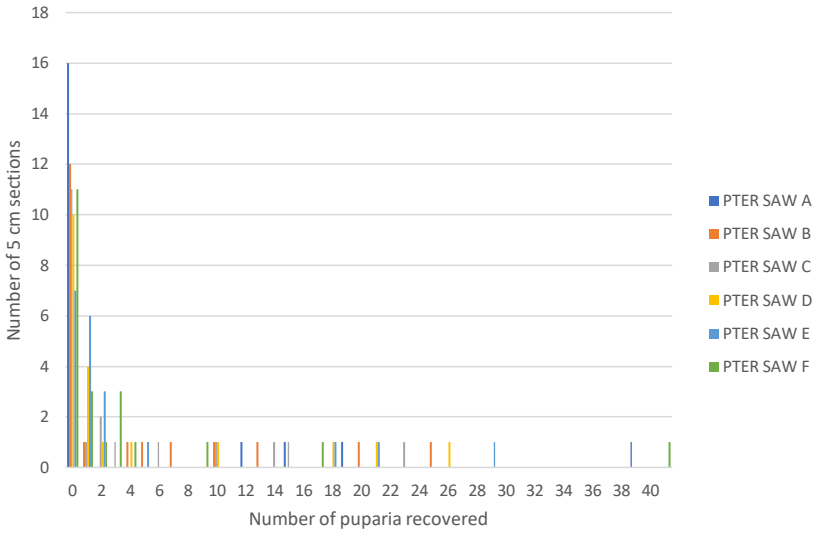


Figure 3.27: The number of 5 cm sections that each number of puparia were recovered from in each experimental run. 'PTER' = *Protophormia terraenovae* and 'SAW' = sawdust substrate. n = 85.

The results of PTER SAW A-F look similar. Unlike the experimental runs conducted with *C. vicina, C. vomitoria* and *L. sericata* the puparia were not recovered throughout the length of the gutter. In fact, the puparia appear to be confined to approximately the middle third of the gutter, almost creating a normal distribution. However, closer inspection of each graph shows that the distribution of puparia may be an oscillating pattern. Again, the frequency graph (Figure 3.27) shows that the number of puparia recovered from each 5 cm section of the gutter was not consistent throughout the experimental runs. Again, the ratios of variance to the mean (Table 3.22) conducted on the results from each experimental run produced a ratio much greater than

one, suggesting that the distribution of puparia recovered was highly clumped. All of the analyses may provide further evidence of aggregation prior to pupariation. The results are discussed in further detail later in this Section.

To summarise, visual inspection of the data in each of the figures (Figures 3.12.a-g, 3.14.a-l, 3.16.a-k, 3.18.a-e, 3.20a-e, 3.22.a-e, 3.24.a-e and 3.26.a-f) clearly shows that the number of puparia recovered from each sampled section is not normally distributed (excluding the experimental runs conducted using *P. terraenovae*). Therefore, the primary alternative hypothesis, for the first set of hypotheses looking at the distribution patterns of all of the data, can be rejected for each experimental run. The ratios of variance to the mean conducted on the results from each experimental run produced a ratio much greater than one, suggesting that the distribution of puparia recovered, during every run, was highly clumped (Tables 3.15-3.22). The frequency graphs of each experiment further show this, as the frequency at which different numbers of puparia were recovered from each 5 cm section was not constant for any of the experimental runs. Therefore, the first null hypothesis can be rejected for all of the runs. Thus, the results of these experimental runs may allow the acceptance of the secondary alternative hypothesis, i.e. a random distribution is most likely occurring during every run. However, similar to the results of the 6 m gutter experiment (Section 3.3.1), the patterns of the distribution of puparia throughout all experimental runs (except the runs conducted with no substrate, where puparia were concentrated at either end of the gutter) appears to be oscillating, not random. Due to the oscillation pattern occurring almost ubiquitously throughout these results, the author proposes that larval aggregation prior to pupariation may be occurring. This theory is discussed in more depth in Section 3.4.

3.3.2.1 One metre horizontal dispersal experiment: substrate

The second set of hypotheses considered any differences in the distribution of puparia shown between the different dispersal substrates. The ratios of variance to the mean tests conducted on all of the experimental runs produced ratios much greater than one (Tables 3.15-3.22). Visual inspection of the graphs suggest that the only difference is between the bare (no substrate) and the other dispersal substrates. The puparia recovered from these experimental runs were mainly recovered from either end of the gutter, very few from the middle sections. This observation is supported by the frequency graphs (Figures 3.17 and 3.21), as the most common number of puparia recovered in each section was 0, in contrast to the frequency graphs of the other substrates where this was not the case. An extreme example of this is the LSER BAR A run, where

45 out of the 50 puparia (90 %) were recovered from one 5 cm section at the end of a gutter. Table 3.23 further demonstrates the differences seen between the bare (no substrate) and all of the other substrates with *C. vicina*. Table 3.23 compares the mean number of 5 cm sections with over 10 and 15 % of puparia recovered, the percentages were chosen as they most clearly show the differences between the distribution of puparia in the different substrates compared to no substrate. There was a higher mean number of sections where over 10 % and 15 % of the puparia were recovered during the experimental runs. This highlights the effect that bare (no substrate) has on the dispersal of post-feeding larvae prior to pupariation, as either significantly high numbers of puparia were recovered or no puparia were recovered from each 5 cm section. There was a slight difference observed in the sawdust substrate compared with the soil substrates, as there appeared to be a small gap in the middle of the gutter where less puparia were recovered and thus a slight tendency for puparia to be recovered closer to the ends of the gutters. Perhaps this difference is due to the lighter density of sawdust compared with commercial soil, 0.066 g/cm^3 and 0.47 g/cm^3 respectively, thus the distribution of puparia in the sawdust substrate produced some minor similarities with bare (no) substrate. However, further examination of this phenomenon produced no significant differences between sawdust and the other substrates (Table 3.23). The null hypothesis that there were no differences observed between the different dispersal substrates can be rejected and the alternative accepted, as there was a clear difference in the distribution pattern observed from the bare (no substrate) when compared with all of the other dispersal substrates.

Table 3.23: The mean number of sections, per experimental run, with over 10 and 15 % of puparia recovered from the 5 cm sections. 'CVIC' = *Calliphora vicina*. 'WLG' = Wildlife Garden soil substrate, 'SAW' = sawdust substrate, 'BAR' = bare (no substrate) and 'COM' = commercial soil substrate. The experimental runs used for this comparison: CVIC WLG A-G, CVIC SAW A-L, CVIC BAR A-K and CVIC COM A-E.

Species	**Substrate**	**Mean number of sections with > 10 % of puparia recovered**	**Mean number of sections with > 15 % of puparia recovered**
CVIC	WLG	0	0.3
CVIC	SAW	0	1.8
CVIC	BAR	2.7	2.9
CVIC	COM	0	1.6

3.3.2.2 One metre horizontal dispersal experiment: species

The final set of hypotheses examined the differences in the distribution pattern exhibited between species. Direct visual comparison of each experimental run conducted with the same dispersal substrate, but different species, shows little observable differences (Figures 3.14.a-l and 3.26.a-f, 3.16.a-k and 3.20.a-e, 3.18.a-e and 3.22.a-e and 3.24.a-e). There were also limited differences between the frequency graphs. However, the graphs of *P. terraenovae* in sawdust appear on visual inspection to be normally distributed and they differed from the results of the only other species tested in sawdust, *C. vicina* (Table 3.24). A Shapiro-Wilk test was carried out on each of the datasets PTER SAW A-F and the *p*-values were all < 0.0001 (W = 0.50555, W = 0.66038, W = 0.71335, W = 0.60202, W = 0.56339, W = 0.4971 respectively). Therefore, all of the runs were highly significantly not normally distributed. Nevertheless, Table 3.23 shows that there was a difference in the distribution pattern of *P. terraenovae* when compared with *C. vicina* in the sawdust substrate. The distribution pattern for each species throughout the 1 m gutter is not even, however there appears to be an opposite pattern observed when the mean percentage of puparia, in each experimental run, recovered from approximately each third of the gutter is examined. For *C. vicina* significantly less puparia were recovered in the middle third of the 1 m gutter, when compared with the first and last third. For *P. terraenovae*, highly significantly more puparia were recovered from the middle section of the 1 m gutter, when compared with the first and last thirds. Therefore, as they hypothesised varying degrees of similarities between all of the species, the null hypothesis, primary and secondary alternative hypotheses (Section 3.1.1.1) can not be accepted and H_3 must be accepted as there is a clear difference in the distribution pattern and over all distance dispersed of *P. terraenovae* in comparison to the other species. Although the results for all species are significantly not evenly or normally distributed, there is a significant difference in the pattern of distribution of the *P. terraenovae* puparia recovered compared with *C. vicina*.

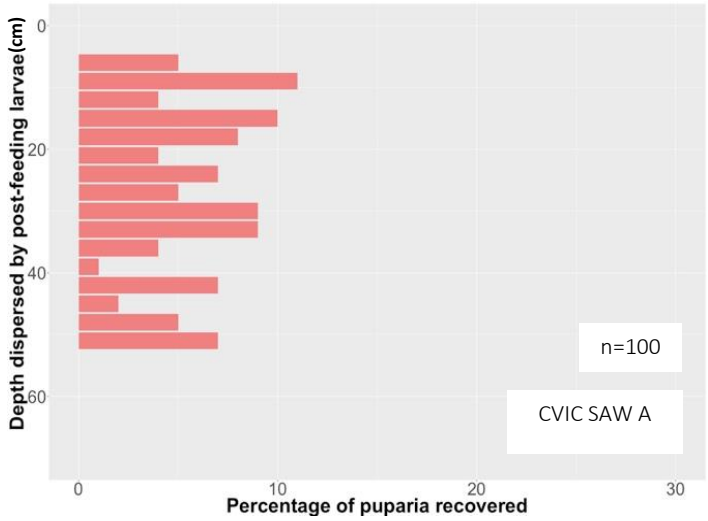
Table 3.24: A comparison of the mean percentage of puparia recovered from ~ each third of the 1 m gutter in the sawdust substrate between CVIC and PTER. 'CVIC' = *Calliphora vicina* and 'PTER' = *Protophormia terraenovae*. Experimental runs used for this comparison: CVIC SAW A-L and PTER SAW A-E.

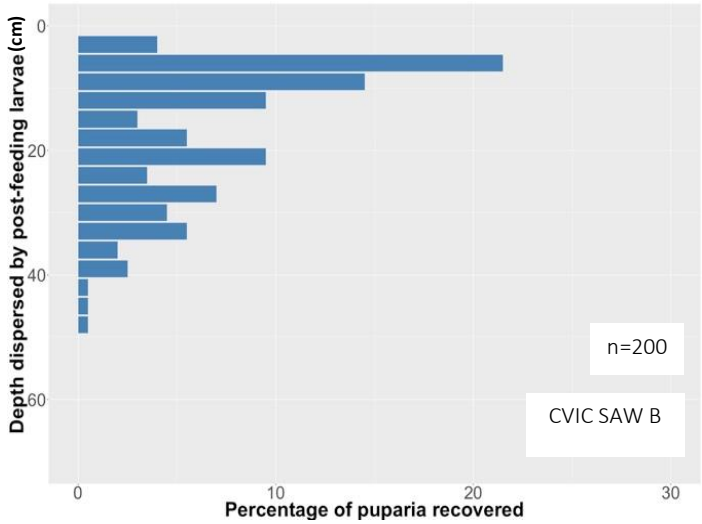
Species	% of puparia in 5 - 35 cm	% of puparia in 40 - 65 cm	% of puparia in 70 – 100 cm
CVIC	42	19	39
PTER	11	84	5

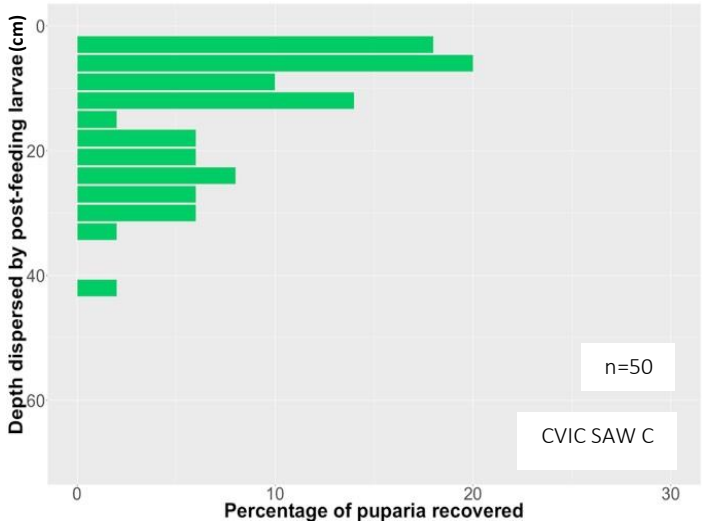
3.3.3 Vertical dispersal experiment

This Section provides an account of six experimental runs conducted using *C. vicina* post-feeding larvae. Two different dispersal substrates were used: commercial topsoil and sawdust. These experiments were conducted to examine any differences in the distribution pattern and depth burrowed of the puparia recovered, depending on the substrate. The larvae were only able to burrow down (no significant horizontal dispersal was possible [the diameter of the pipe was 8 cm]) and therefore the depth and distribution pattern of puparia resulting from these restricted post-feeding larvae was examined.

The results of these runs are represented in Figures 3.28.a-c and 3.29.a-c. In order to examine the distribution of the puparia recovered from each section further, ratio of variance to the mean and skewness tests were carried out in RStudio®.







Figures 3.28.a-c: The percentage of puparia recovered from each 3 cm section (24 sections in total) from each experiment conducted in a pipe. 'CVIC' = *Calliphora vicina* and 'SAW' = sawdust substrate. The number of individuals used in each experiment is shown on each

Table 3.25: Summary of the ratios of variance to the mean conducted on each experimental run, where a score > 1.0 indicates aggregation and a score closer to 1 suggests a Poisson distribution. 'CVIC' = *Calliphora vicina* and 'SAW' = sawdust substrate.

	N	Ratio of variance to the mean
CVIC SAW **A**	100	1.70
CVIC SAW **B**	200	11.56
CVIC SAW **C**	50	2.83

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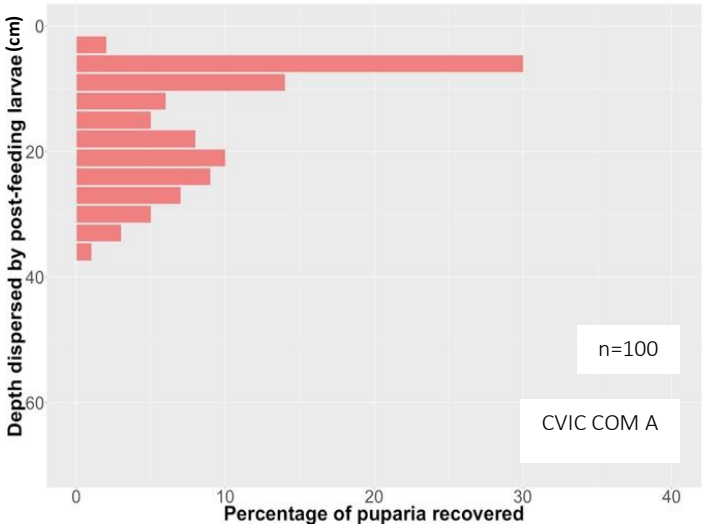
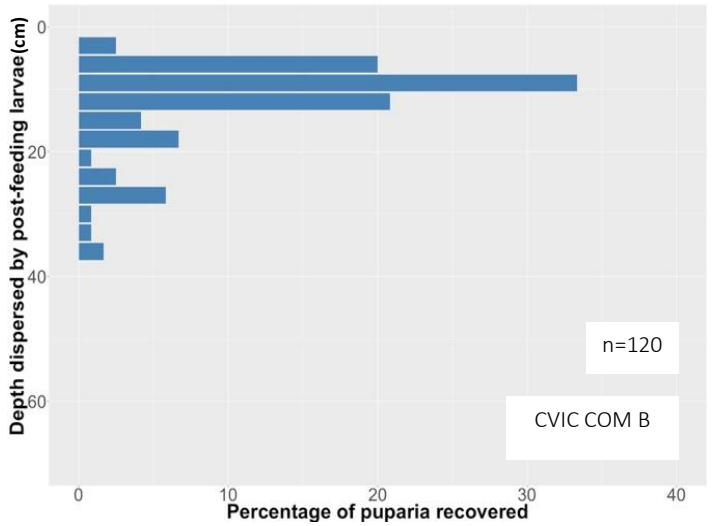
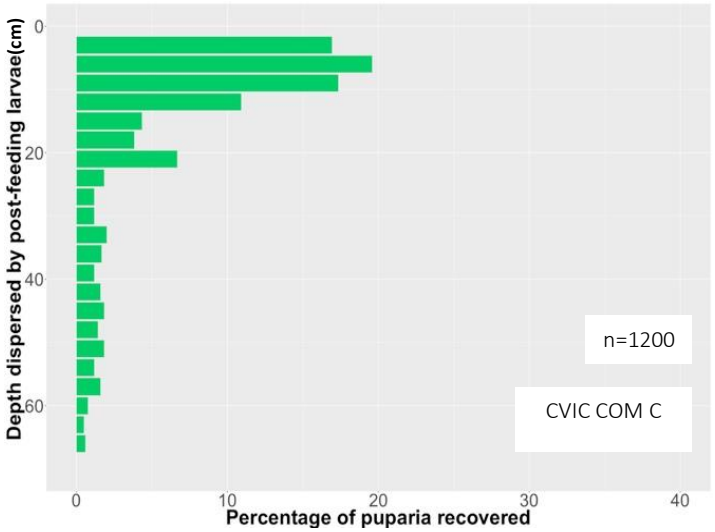
Visual inspection of the data of the experimental runs conducted in sawdust suggests that the results are similar. In all cases puparia were recovered from up to 50 cm deep. The mean depth dispersed in CVIC SAW B was 15 cm and CVIC SAW C was 15 cm, however CVIC SAW A was 27 cm. Moreover 42 % of puparia in CVIC SAW A, 68 % in CVIC SAW B and 76 % in CVIC SAW C were recovered from the top 20 cm of the dispersal substrate. The distribution pattern of CVIC SAW A appears almost even with oscillating numbers of puparia recovered. However, this oscillation pattern does not look as apparent as it did in the horizontal experiments (Sections 3.3.1 and 3.3.2). The distribution pattern of CVIC SAW B and C appear to be positively skewed towards the origin, with oscillating numbers of puparia recovered. The ratios of variance to the mean (Table 3.25) conducted on the results from each experimental run produced a ratio much greater than one, suggesting that the distribution of puparia recovered was highly clumped. The results are discussed in further detail later in this Section.







Figures 3.29.a-c: The percentage of puparia recovered from each 3 cm section (24 sections in total) from each experiment conducted in a pipe. 'CVIC' = *Calliphora vicina* and 'COM' = commercial topsoil substrate. The number of individuals used in each experiment is shown on each graph by 'n'.

Table 3.26: Summary of the ratios of variance to the mean conducted on each experimental run, where a score > 1.0 indicates aggregation and a score closer to 1 suggests a Poisson distribution. 'CVIC'= *Calliphora vicina* and 'COM' = commercial topsoil substrate.

	N	Ratio of variance to the mean
CVIC COM **A**	100	14.10
CVIC COM **B**	120	17.31
CVIC COM **C**	1200	96.05

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Visual inspection of the data of the experimental runs conducted in commercial soil suggests that the results are similar. In CVIC COM A and B puparia were recovered from up to 40 cm deep, but in CVIC COM C puparia are recovered up to 65 cm deep. In comparison to the experimental runs conducted in sawdust, the mean depth of dispersal for all runs in commercial soil was very similar; CVIC COM A was 15 cm, in CVIC COM B was 12 cm and in CVIC COM C was 16 cm. The average depth dispersed in CVIC COM C is important to note because n was much higher in this experiment, by a factor of x10. As previously stated in horizontal dispersal, (Section 3.3.1) it was determined that n was not a factor that affected dispersal, and this experimental run highlights that. Therefore, the fact that even with a high value of n (i.e. 1200 larvae), the average depth dispersed was still comparable to that with the much lower values of n (i.e. 100 or 120 larvae) may show that higher larval densities do not necessarily increase the mean vertical dispersal depth, at least to the densities tested here. Moreover 75 % of puparia in CVIC COM A, 88 % in CVIC COM B and 80 % in CVIC COM C were recovered from the top 20 cm of the dispersal substrate. Again, a similar percentage of puparia were recovered from CVIC COM C, compared with the other two runs. The distribution patterns all of the experimental runs looked alike and were positively skewed towards the origin. The puparia recovered did not appear to create an oscillating pattern, as was seen in CVIC SAW A-B. The ratios of variance to the mean (Table 3.26) conducted on the results from each experimental run produced a ratio much greater than one, suggesting that the distribution of puparia recovered was highly clumped. The results are discussed in further detail later in this Section.

3.3.3.1 Vertical dispersal experiment: substrate

The first set of hypotheses examined the ability of *C. vicina* post-feeding larvae to successfully burrow to different depths of the dispersal substrate. The null hypothesis can be rejected and

the alternative accepted as the majority of the puparia were recovered more than 5 cm deep in each experimental run:

- CVIC SAW A - > 50 % puparia recovered from the top 27 cm of substrate, the deepest that puparia were recovered was 51 cm deep.
- CVIC SAW B - > 50 % puparia recovered from the top 15 cm of substrate, the deepest that puparia were recovered was 48 cm deep.
- CVIC SAW C - > 50 % puparia recovered from the top 12 cm of substrate, the deepest that puparia were recovered was 42 cm deep.
- CVIC COM A - > 50 % puparia recovered from the top 12 cm of substrate, the deepest that puparia were recovered was 36 cm deep.
- CVIC COM B - > 50 % puparia recovered from the top 9 cm of substrate, the deepest that puparia were recovered was 36 cm deep.
- CVIC COM C - > 50 % puparia recovered from the top 9 cm of substrate, the deepest that puparia were recovered was 66 cm deep.

3.3.3.2 Vertical dispersal experiments: species

The second set of hypotheses examined the distribution pattern of the puparia recovered. Visual inspection of Figures 3.28.a-c and 3.29.a-c show that the distribution is not even, moreover the results of the ratios of variance to the mean outlined in Tables 3.25 and 3.26 all give values much greater than one which further suggests that this is the case. Again, visual inspection of the results suggests that the puparia were not recovered in a random distribution, instead the patterns appear to all be positively skewed. As such, all the datasets were tested for skewness. Testing for skewness examines the symmetry of the data distribution, i.e. a skewness value of 0 shows perfectly symmetrical data (a normal distribution), a negative value shows a negative skew (away from the origin) and a positive value shows a positive skew (towards the origin). A skewness value of less than -1 or more than 1 shows the distribution is highly skewed. The results of the skewness test are summarised below:

- CVIC SAW A: 0.1426494
- CVIC SAW B: 1.735816
- CVIC SAW C: 1.320874
- CVIC COM A: 2.343008
- CVIC COM B: 2.245479
- CVIC COM C: 1.650299

There was a strong positive skew in all experimental runs apart from CVIC SAW A. Thus, the null (even distribution) and second alternative hypotheses (random distribution) can be rejected and the primary alternative hypothesis (positive skew), accepted.

There appears to be no difference exhibited by the post-feeding larvae dependent on substrate; the average depth burrowed in all experimental runs was very similar (apart from CVIC SAW A, where the mean depth burrowed was 27 cm). There is no visual difference between the graphs (Figures 3.27.a-c and 3.28.a and b [the depth dispersed in Figure 3.28 c was clearly more than the other runs, but mean depth dispersed was comparable]), no difference between the ratios of variance to the mean values (Tables 3.25 and 3.26), no differences between the post-feeding larval ability to disperse vertically and no difference between the skewness of the puparia recovered in the two substrates (again, CVIC SAW A was the only experimental run that was not strongly positively skewed).

Although the results of CVIC SAW A appear quite different to the results of the other experimental runs, on closer inspection the differences are not great. The maximum depth dispersed in CVIC SAW A was 50 cm, which is the same as for SAW B and C. The distribution pattern is only slightly positively skewed (0.1426494), in comparison to the other runs being strongly positively skewed. Although the average depth dispersed was much greater compared with the other runs (27 cm versus 12- 16 cm deep), because the distribution was less positively skewed than that of the other runs.

3.3.4 Simultaneous horizontal and vertical dispersal experiment

This Section provides an account of the results of six experimental runs conducted using *C. vicina* post-feeding larvae, three with commercial topsoil and three with sawdust as the dispersal substrate. This experiment was conducted to examine the horizontal and vertical dispersal of post-feeding larvae simultaneously. The dispersing larvae were not forced to move only horizontally or vertically as in Sections 3.3.1, 3.3.2 and 3.3.3, but rather were given the choice of both dispersal options to determine the outcome in a near-natural situation.

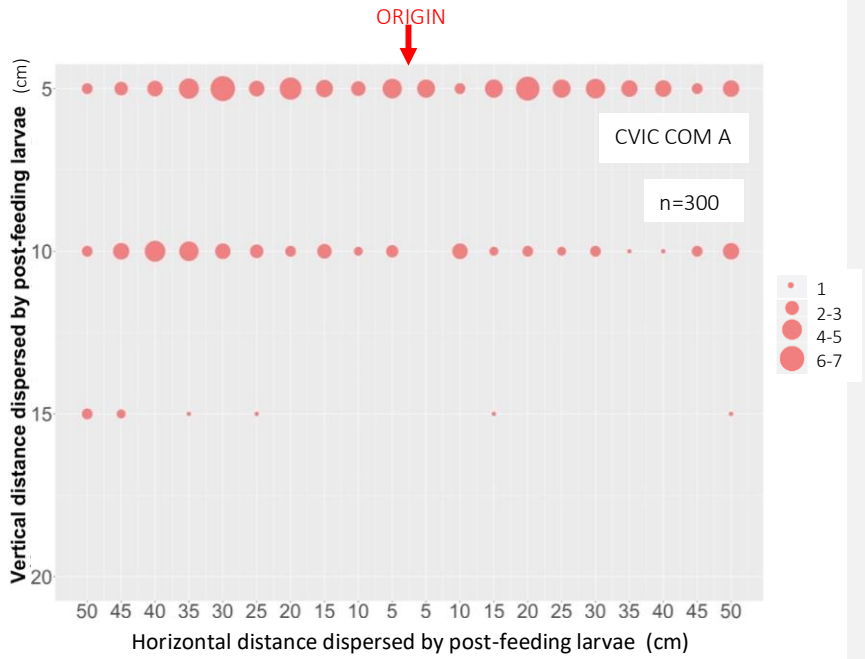


Figure 3.30: The percentage of *Calliphora vicina* (CVIC) puparia recovered from each 5 x 5 cm section (100 sections in total). 'COM' = commercial soil substrate. The size of the circle at each point is proportional to the number of puparia recovered.

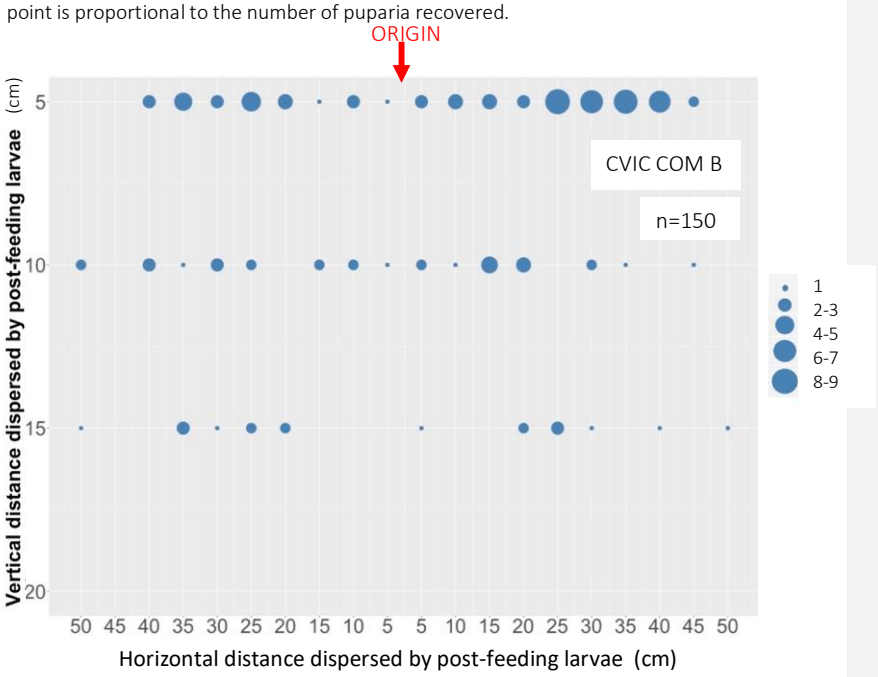


Figure 3.31: The percentage of *Calliphora vicina* (CVIC) puparia recovered from each 5 x 5 cm section (100 sections in total). 'COM' = commercial soil substrate. The size of the circle at each point is proportional to the number of puparia recovered.

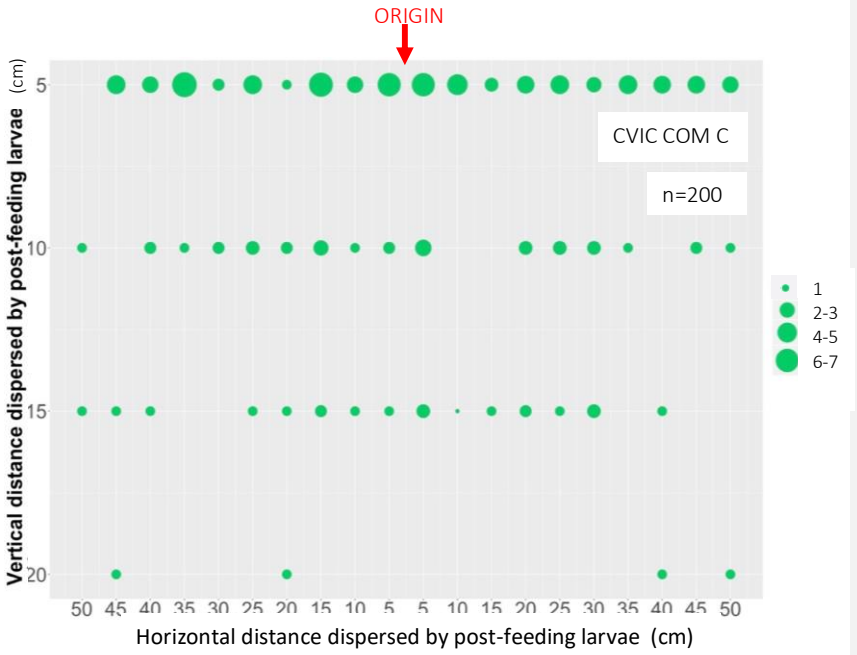


Figure 3.32: The percentage of *Calliphora vicina* (CVIC) puparia recovered from each 5 x 5 cm section (100 sections in total). 'COM' = commercial soil substrate. The size of the circle at each point is proportional to the number of puparia recovered.

Initially the results of CVIC COM A-C appear slightly different. In CVIC COM A very few puparia were recovered from 15 cm deep (3 %), in CVIC COM B slightly more puparia were recovered from 15 cm deep (12 %) and in CVIC COM C more puparia were recovered from 15 cm deep (10 %) and some from 20 cm deep (3 %). However, in all three experimental runs approximately 60 % of puparia were recovered from the top 5 cm. The horizontal distribution pattern in each experimental run does not appear even, but oscillating, similar to the results of Section 3.3.1 and 3.3.2. These results may provide further evidence of aggregation prior to pupariation. The results are discussed in further detail later in this Section.

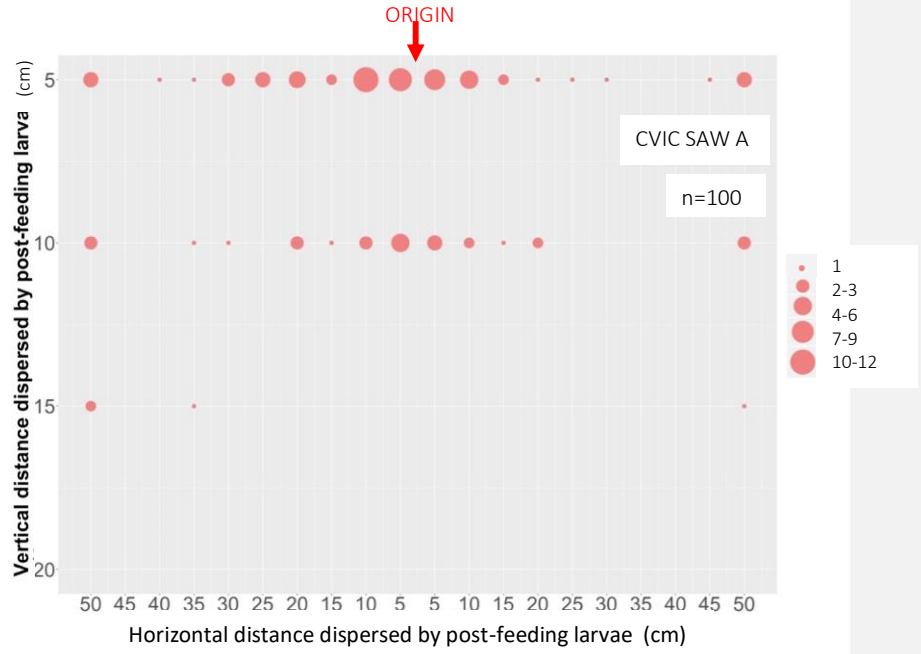


Figure 3.33: The percentage of *Calliphora vicina* (CVIC) puparia recovered from each 5 x 5 cm section (100 sections in total). 'SAW' = sawdust substrate. The size of the circle at each point is proportional to the number of puparia recovered.

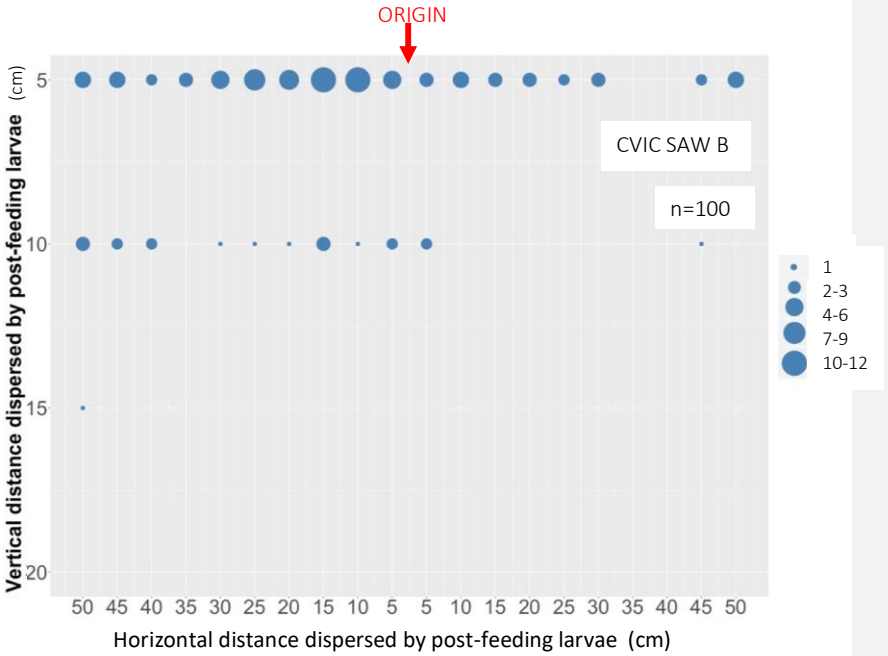


Figure 3.34: The percentage of *Calliphora vicina* (CVIC) puparia recovered from each 5 x 5 cm section (100 sections in total). 'SAW' = sawdust substrate. The size of the circle at each point is proportional to the number of puparia recovered.

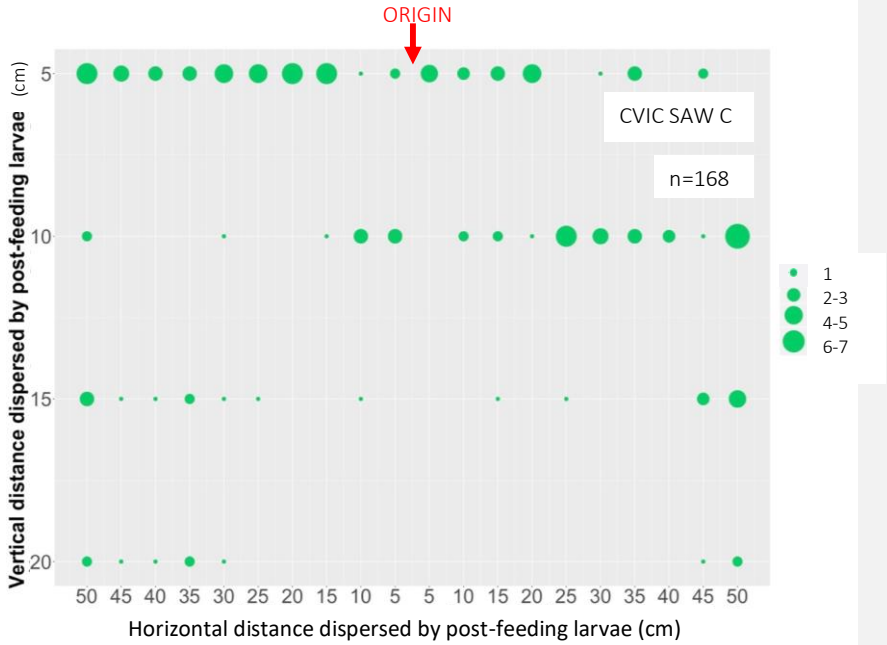


Figure 3.35: The percentage of *Calliphora vicina* (CVIC) puparia recovered from each 5 x 5 cm section (100 sections in total). 'SAW' = sawdust substrate. The size of the circle at each point is proportional to the number of puparia recovered.

The results of CVIC SAW A and B look similar, while CVIC SAW C looks slightly different. In both CVIC SAW A and B very few puparia were recovered from 15 cm deep (4 % and 1 % respectively), whereas in CVIC SAW C more puparia were recovered from 15 cm deep (13 %) and some were recovered from 20 cm deep (6 %). However, again in all three experimental runs over 50 % of puparia were recovered from the top 5 cm. Again, the horizontal distribution pattern in each experimental run does not appear even, but oscillating, similar to the results of Section 3.3.1 and 3.3.2. These results may provide further evidence of aggregation prior to pupariation. The results are discussed in further detail later in this Section.

Visual inspection of all of the data (Figures 3.29-3.34) clearly show that there was not an even distribution of puparia recovered from the entire experimental apparatus. In fact, in all cases at least half of the puparia were recovered from the top 5 cm:

- CVIC COM A: 63 % of the puparia were recovered from the top 5 cm.
- CVIC COM B: 57 % of the puparia were recovered from the top 5 cm.
- CVIC COM C: 66 % of the puparia were recovered from the top 5 cm.
- CVIC SAW A: 66 % of the puparia were recovered from the top 5 cm.
- CVIC SAW B: 80 % of the puparia were recovered from the top 5 cm.
- CVIC SAW C: 50 % of the puparia were recovered from the top 5 cm.

The mean percentage of puparia recovered from the top 5 cm with the commercial soil substrate was 62.0 % and the mean percentage of puparia recovered from the top 5 cm with the sawdust substrate was 65.3 %. As the majority of puparia were recovered from the top 5 cm of dispersal substrate in all cases, the null and second alternative hypotheses can be rejected and the primary alternative hypothesis accepted. Moreover, when these results are compared to the results of the vertical dispersal experiments (Section 3.3.3), it is clear that there was also a lower density of puparia recovered per cm^3 during these experimental runs (Table 3.27). This further shows the preference for horizontal dispersal over vertical dispersal as the highest number of puparia recovered from all experimental runs in the vertical dispersal and the simultaneous horizontal and vertical experimental runs were in the top 6 cm or 5 cm, respectively. The mean highest density of puparia recovered per cm^3 (based on the number of puparia per unit volume (cm^3)) during the vertical experimental runs was 0.41 and for the simultaneous horizontal and vertical experimental runs was 0.090 (calculated from the data presented in Table 3.26).

Table 3.27: The highest density of puparia recovered per cm^3, based on the highest number of puparia recovered in one section, during each experimental run, per unit volume. The results of the vertical experiments are outlined in Section 3.3.3 and the results of the simultaneous vertical and horizontal experiments are outlined in this section. The highest number of puparia recovered in one section during the vertical and simultaneous vertical and horizontal experiments occurred in the top 6 cm and 5 cm, respectively.

Experiment type	Experiment	Highest density of puparia recovered per cm^3
Vertical	CVIC SAW A	0.073
Vertical	CVIC SAW B	0.28
Vertical	CVIC SAW C	0.066
Vertical	CVIC COM A	0.20
Vertical	CVIC COM B	0.26
Vertical	CVIC COM C	1.6
Simultaneous horizontal and vertical	CVIC SAW A	0.096
Simultaneous horizontal and vertical	CVIC SAW B	0.0080
Simultaneous horizontal and vertical	CVIC SAW C	0.10
Simultaneous horizontal and vertical	CVIC COM A	0.17
Simultaneous horizontal and vertical	CVIC COM B	0.096
Simultaneous horizontal and vertical	CVIC COM C	0.11

During the experimental runs outlined in this section, over 62 % of puparia were recovered from the top 5 cm of the experimental apparatus during all experimental runs, in comparison, during the vertical experiment the majority (> 50 %) of puparia were recovered from over 5 cm deep. This reiterates the clear difference between the results and thus the tendency for larvae to disperse horizontally over vertically.

There appears to be no difference exhibited by the post-feeding larvae dependent on substrate. There is little visual difference in the graphs (Figures 3.29-3.34 and no difference in post-feeding larval preference for horizontal over vertical dispersal: the mean percentage of puparia recovered from the top 5 cm of the apparatus for commercial soil and sawdust is very similar, 62.0 % and 65.3 %, respectively.

3.4 Discussion

This Chapter has examined the horizontal and vertical dispersal of *Calliphora vicina, C. vomitoria, L. sericata* and *P. terraenovae*, with the main focus on *C. vicina*. The main aspects that have been studied are: 1) the ability of post-feeding larvae to disperse horizontally and vertically; 2) the distribution pattern of the resulting puparia; 3) how different dispersal substrates affect the behaviour and distribution patterns of the post-feeding larvae; and 4) any differences exhibited between species.

In all of the experimental runs, conducted using *C. vicina,* outlined in Section 3.3.1 in a 6 m gutter, over half of the puparia were recovered within 4 m of the post-feeding larval origin (the end of the gutter) (the results of the experimental runs conducted with *P. terraenovae* are discussed separately, later in this Section). The post-feeding larvae were able to disperse unimpeded, but in a single direction, throughout the full 6 m length of the gutter and yet in all cases the majority of puparia (> 50 %) were recovered within the first 4 m and moreover, for all experimental runs, apart from CVIC SAW A, the majority of puparia (> 50 %) were recovered within the first 3 m of the gutter. Under extreme conditions where larval burial into the dispersal substrate is impossible, such as the bare plastic dispersal substrate (substrate will be discussed in further detail later in this Section), the larvae tended to disperse the full length of the 6 m gutter. Previous studies have demonstrated that dispersing larvae are able to move up to 50 m from the food source (Byrd and Castner, 2009). Given the results of the bare gutter experimental runs, the dispersing larvae would most likely have dispersed further than the 6 m limit of this experiment. Given a more suitable pupariation environment (two types of soil or sawdust) the dispersing larvae exhibit a preference for limited dispersal. This behaviour makes evolutionary sense, as limiting their energy expenditure when a suitable pupariation medium is available should enable shorter pupariation periods and the emergence of larger adults (Arnott and Turner, 2008; Mai and Amendt, 2012). According to the results of this study, in real-life situations where the substrate is not impenetrable, the majority of larvae will most likely disperse up to 4 m from the food source.

It has previously been noted that in natural outdoor conditions, such as soil, most puparia are recovered from the top 5 cm of their dispersal substrate (Godoy *et al.*, 1995; Cammack *et al.*, 2010). The experiments conducted in Section 3.3.3 examined the ability of larvae to disperse vertically when there is no option for them to disperse horizontally. During all of the experimental runs, over half of the puparia were recovered from a depth of the first 9 cm or more. Moreover, the maximum depth recorded from each experiment was at least 36 cm, and during one

experiment, was 66 cm deep. These experiments show that dispersing post-feeding larvae, when denied the option of horizontal dispersal, are capable of burrowing much deeper than the 5 cm generally reported in the literature (Godoy *et al.*, 1995; Gomes *et al.*, 2006b; Cammack *et al.*, 2010). There may be advantages to this ability, as it has been shown that puparia situated deeper in the substrate are less susceptible to parasitism, by parasitoids like *Nasonia vitripennis* (Frederickx *et al.*, 2014). It is important to note that although the puparia were recovered deeper in the substrate in the CVIC COM C run (Section 3.3.3), compared with the other two runs, the mean depth dispersed and percentage of puparia recovered from the top 20 cm of the substrate was comparable with that of the other runs. Therefore, the difference in the number of larvae introduced produced no forensically relevant effect on the position of puparia in the dispersal substrate.

Section 3.3.4 examined the post-feeding horizontal and vertical dispersal of *C. vicina* larvae simultaneously. The experimental apparatus used in Section 3.3.4 allowed horizontal and vertical dispersal unimpeded and thus simulated a natural outdoor setting better than the previous experimental set-ups (gutter [Section 3.3.1 and 3.3.2] and pipe [Section 3.3.3]). Therefore, the horizontal dispersal of larvae was made without pressure from overcrowding at subsequent pupariation, as clearly, they could have tolerated a much higher density, on average 4.6 times greater (0.41/0.090), even up to 17.8 times greater (1.6/0.090). There was no constraint on vertical dispersal, but larvae instead showed the tendency to disperse horizontally. As suggested in Section 3.3.3, higher larval density does not appear to be a prompt for vertical dispersal and thus a preference for horizontal dispersal over vertical dispersal seems plausible. In sawdust and soil substrates a mean of 62.0 % and 65.3 %, respectively, of puparia were recovered within the top 5 cm of the substrate (Section 3.3.4; CVIC SAW COM A-C AND CVIC SAW A-C). This shows a larval preference for horizontal dispersal over vertical dispersal, when given the option of both. The field experiments (Chapter 4), will examine this concept in a natural outdoor.

From all of the horizontal experiments, the distribution pattern appears to be oscillating with variable amplitude and frequency, with potential aggregate formation prior to pupariation. Moreover, each experimental run was sampled by hand and a more intense aggregation behaviour of larvae was observed when recovering the puparia, more distinct than can be seen in Figures 3.8-3.11. Thus, puparia that were recovered from the same 5 cm or 10 cm sampled section were often found directly touching each other. This suggests a preference of post-feeding larvae to either seek out puparia or to aggregate with other post-feeding larvae prior to pupariation. Aggregation prior to pupariation has been suggested in the literature and the findings of these experiments support that possibility (Reigada and Godoy, 2005; Lewis and

Benbow, 2011). The aggregative distribution of puparia appears to be limited to horizontal dispersal as the results outlined in Section 3.3.3 suggest that the vertical distribution of puparia is not random, but positively skewed towards the surface of the dispersal substrate. This result makes sense as when post-feeding larvae are given the option of horizontal and vertical dispersal, they show a preference for horizontal dispersal (Section 3.3.4). Post-feeding larvae burrow directly into their dispersal substrate prior to horizontal or further vertical dispersal (Gomes *et al.*, 2006a). Therefore, their dispersal is through substrate regardless of the direction, horizontal or vertical, i.e. larvae do not disperse on the surface if they are able to burrow in to the substrate (Lewis and Benbow, 2011). Thus, even though deeper burial has been associated with some advantages such as protection from parasitism (Frederickx *et al.*, 2014), it appears most likely that larval preference for horizontal dispersal is due to the increased difficulty and thus energy expenditure incurred during the emergence from the dispersal substrate of the adult fly if increased vertical dispersal has occurred.

The effect of the dispersal substrate on dispersing post-feeding larvae is barely discussed in the literature (Arnott and Turner, 2008; Robinson *et al.,* 2018). Logically the substrate through which post-feeding larvae are moving, or on which they are dispersing, must have an impact on their speed, energy expenditure and hence, final pupariation site. If a substrate is not suitable for larval penetration and subsequent pupariation, post-feeding larvae have been shown to continue dispersal until an adequate site is found, for example moving up to 26 m on waterlogged soil until dryer, and therefore more suitable, soil was found (Lewis and Benbow, 2011). Surprisingly, in this study, differences were only recorded between the bare plastic dispersal substrate in comparison with the other, more suitable substrates (soil and sawdust). It was originally thought that sawdust would provide an 'easier' medium through which to disperse and therefore differences would be observed between sawdust and soil. However, this was not the case. In Sections 3.3.1 and 3.3.2 the difference between dispersal substrate on the behaviour of post-feeding larvae and subsequently the position of the recovery of puparia was observed. In the 6 m gutter experiment (Section 3.3.1) over half of the puparia in the bare plastic runs were recovered from the first and last 50 cm of the gutter, a significant difference when compared with the other substrates, where the majority of larvae were recovered from within the first 4 m only. Essentially the post-feeding larvae, in the bare plastic runs, dispersed the full 6 m of the gutter in search of a suitable pupariation site, and pupariated mainly at either end. This is possibly due to the thigmotactic response of post-feeding larvae (Holmes *et al.*, 2013), whereby the larvae concentrate at the ends of the gutter, where the chance of contact with more edges of the gutter is greatest. This is even more clear when examined in conjunction with the results from the bare plastic experiments where strong puparial aggregates were formed in every experimental run conducted

with no dispersal substrate (Section 3.3.1 [CVIC BAR A-C] and Section 3.3.2 [CVIC BAR A-K and LSER BAR A-E]).

Lastly, the differences in larval behaviour prior to pupariation, with regards to species was examined. It was determined that species exhibited no differential behaviour under all circumstances, apart from *P. terraenovae*. In the sawdust substrate, over 60 % of puparia (almost 100 % in PTER SAW B) were recovered within the first 50 cm of the 6 m gutter for two of the experimental runs (Section 3.3.1, PTER SAW B and PTER SAW C), compared to less than 10 %, 10 % and 45 % in CVIC SAW A, CVIC SAW B and CVIC SAW C, respectively (Section 3.3.1, Table 3.14). The results in the 1 m gutter of PTER SAW A-E when compared with CVIC SAW A-L (Section 3.3.2) corroborate the results of *P. terraenovae* in the 6 m gutter as their distribution pattern suggests limited horizontal dispersal. There were significantly more *P. terraenovae* puparia recovered from the middle section of the 1 m gutter, versus the first and last thirds. In comparison, significantly less *C. vicina* puparia were recovered from the middle third of the 1 m gutter, versus the first and last third. *Protophormia terraenovae* is known not to disperse far from their feeding substrate and they are, in fact, often recovered as puparia on the feeding substrate itself (Erzinçlioğlu, 1996; Pohjoismäki *et al.*, 2010). Therefore, these results were not surprising. However, the results of the first experiment using *P. terraenovae* (Section 3.3.1., PTER SAW A) were very interesting as puparia were recovered up to 5.5 m from the origin and the distribution pattern of the puparia appeared much more similar to those of the other species. It is an interesting result, as there were no other differences (e.g. temperature, humidity, number of larvae, age of larvae etc) between the three experimental runs (PTER SAW A-C) that could account for the difference in the distribution pattern of the puparia and the extended dispersal of the post-feeding larvae in PTER SAW A. Therefore, contrary to current knowledge, *P. terraenovae* are capable of dispersing as far as the other UK blow fly species tested here, although they only appear to do so rarely (Section 3.3.1).

Chapter 4: Field experiment

4.1 Introduction

The previous Chapters have examined the main factors that affect, and are affected by, post-feeding larval dispersal in a controlled laboratory setting. In this way the different factors were examined individually, and the results recorded likewise. The experiments were carried out under controlled conditions in order to determine exactly what factors (e.g. different dispersal substrates) affect the horizontal and vertical dispersal of post-feeding calliphorid larvae. However, in a real casework situation no factors occur independently. Therefore, creating an experiment to consider all factors together in the field under 'real' conditions is essential to validate the laboratory data. Therefore this section outlines an experiment that was conducted in the Wildlife Garden of the Natural History Museum, London (Ware *et al.*, 2016).

During the simultaneous horizontal and vertical controlled experimental runs, an average of 62 % of puparia were recovered from the top 5 cm of the apparatus, with commercial soil substrate (Section 3.3.4). Therefore, it was hypothesised that the majority of larvae would also be collected from the top 5 cm of the substrate during the field experimental runs (Section 3.3.4).

4.1.1 Field experiment hypotheses

There were three sets of hypotheses determined for the field experiment, each focusing on a different factor:

Vertical dispersal:
H_0: There would be an even distribution of puparia recovered from 0-5 cm, 5-10 cm and so on.
H_1: The majority of puparia would be recovered from the top 5 cm of substrate, less than 10 % from the next 5 cm and very few at a lower depth, supporting the results of the controlled experiments (Section 3.3.4).

Horizontal dispersal:
H_0: The majority, > 65 %, of the puparia would be recovered within a 4 m radius from the point of origin (as seen in Section 3.3.1, a mean of 83 % [68, 88 and 93 %] of puparia were recovered within the first 4 m during the experiments that used the Wildlife Garden dispersal substrate and the larvae have only horizontal dispersal in one direction as an option).

H_1: The majority, > 65 %, of the puparia would be recovered from more than a 4 m radius from the point of origin.

Distribution of puparia:

H_0: The puparia from the site would be recovered in an even distribution.

H_1: The puparia would be recovered in a random distribution.

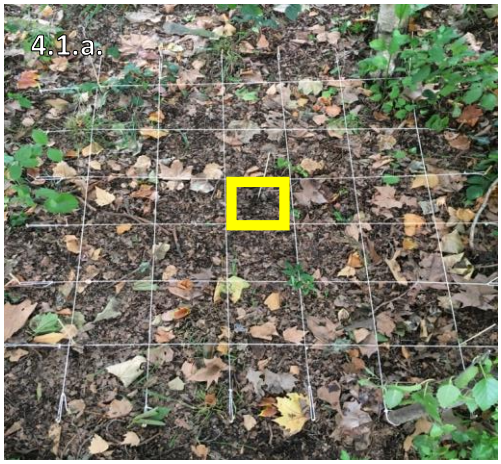
H_2: The puparia would be recovered in a '3D' normal distribution about the point of origin.

4.2 Methodology

4.2.1 Field experiment

Colonies of *Calliphora vicina* were established and reared, as outlined in Section 2.2.1. Post-feeding *C. vicina* larvae were introduced to selected areas of the Wildlife Garden. The number of larvae added to each experimental run varied depending on how many *C. vicina* had been reared at the time, 600 larvae for the first experimental run, 200 for the second and 700 for the third. All post-feeding larvae were introduced to a square area of 5 x 5 cm^2, on the surface of the topsoil. A 1 m x 0.5 m x 0.5 m metal 'dog' cage was placed on top of the experimental area such that the larvae were situated in the middle of the bottom floor of the cage. The cage ensured that natural vertebrate scavengers of the garden would be unable to disturb the larvae (Hofer *et al.*, 2017). A Tinytag Plus 2® Gemini Data Logger set to record hourly data, was placed in the corner of the cage to record ambient temperature and humidity. The ambient and soil temperatures in the Wildlife Garden were also compared (Figure 4.8) using Tinytag Plus 2® Gemini Data Loggers.

The larvae were left for 10 days to disperse and pupariate. After 10 days, the cage was removed and the 1 m^2 experimental area was sampled in 25 sections of 20 cm x 20 cm sections. The areas were measured and sectioned off using string and wooden spikes (Figure 4.1.a. and 4.1.b.). Each section was sampled 5 cm deep; where ≥ 10 puparia were found in the top 5 cm, the section was sampled another 5 cm deep until < 10 puparia were recovered at each 5 cm depth. Three control 20 cm x 20 cm sections were sampled 1 m from the edge of the area adjacent to the quarter from which most puparia were collected (Figure 4.1.b.). The control areas were sampled to establish a baseline of puparia already present in the soil. The soil of the experimental area was not sampled prior to each experiment, to ensure that the soil was not disturbed from its natural state of compaction.



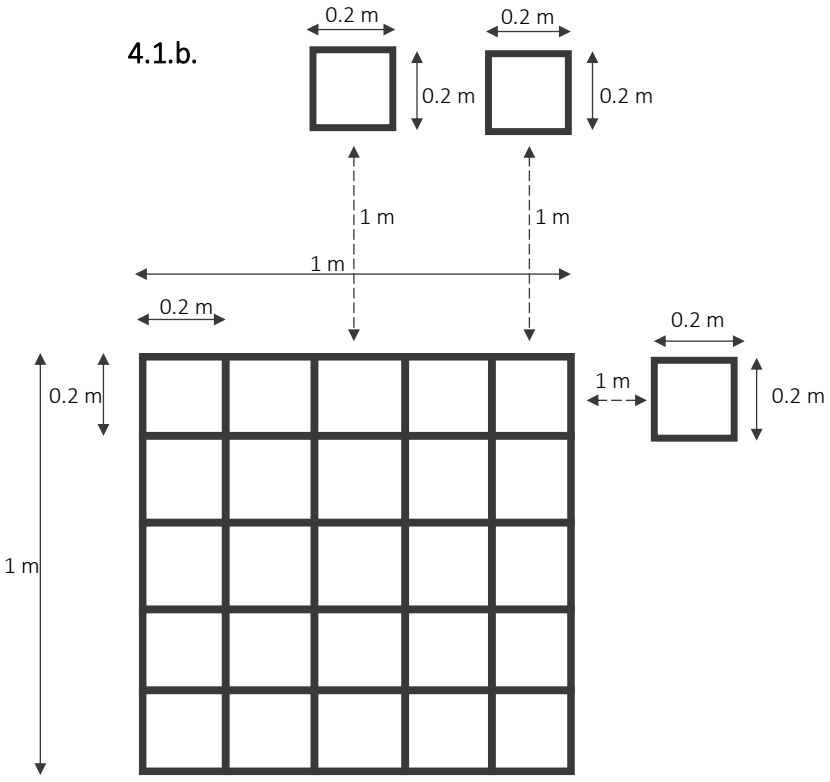


Figure 4.1.a: The 1 m ^{2} sampling area of the experimental space, where each square is 0.2 m ^{2}. The yellow box represents the central square where the larvae were introduced.
Figure 4.1.b: The measurements of the sampling area, including the three control sample squares.

Three runs of the experiment were conducted. Each run was carried out in the same general area of the Wildlife Garden to ensure that the dispersal substrate was similar (Figure 4.2.a. and 4.2.b.). However, each run was conducted at least 3 m from the other runs to ensure that there was no possibility of contamination of the soil with ground marking signals (i.e. chemical trails left by previous dispersing larvae) (Boulay *et al.*, 2016) and to retain the natural compaction and state of the soil (Figure 4.2.a. and 4.2.b.). As there were never any puparia recovered from the control samples, 3 m was determined as a sufficient distance.

The counts of puparia recovered from each sampled square and the other parameters, temperature and humidity, were recorded were analysed using RStudio®.

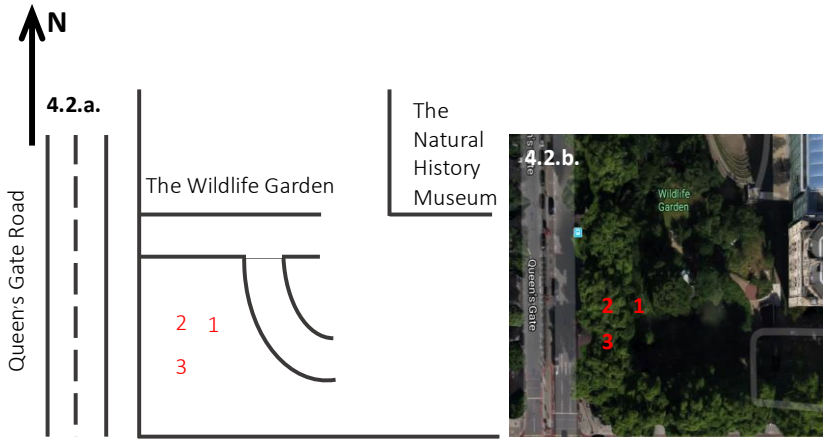
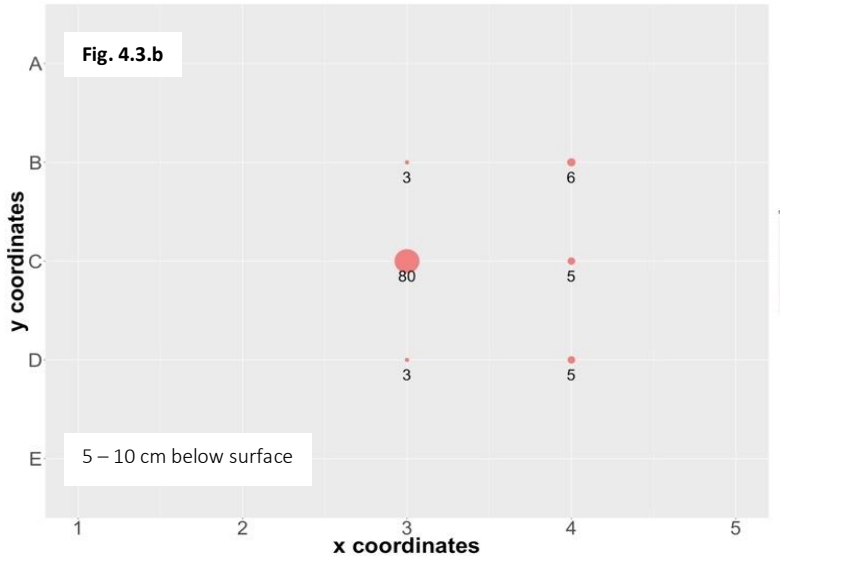


Figure 4.2.a: The position of the experimental areas of the three runs of this experiment (1, 2 and 3) in the Wildlife Garden of the Natural History Museum (not to scale). **Figure 4.2.b:** Aerial view of the Wildlife Garden, showing the three positions of each run of this experiment, under the tree canopy (Google Maps 2017). The direction of North is shown.

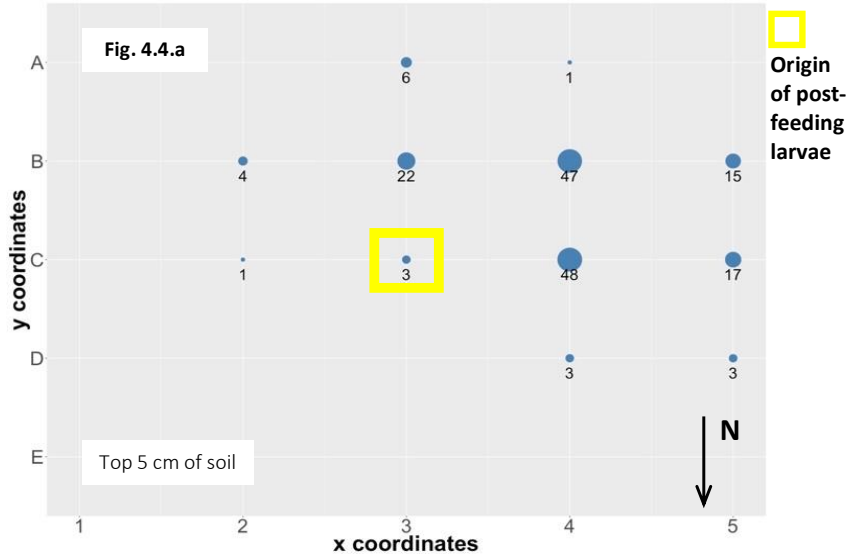
4.3 Results

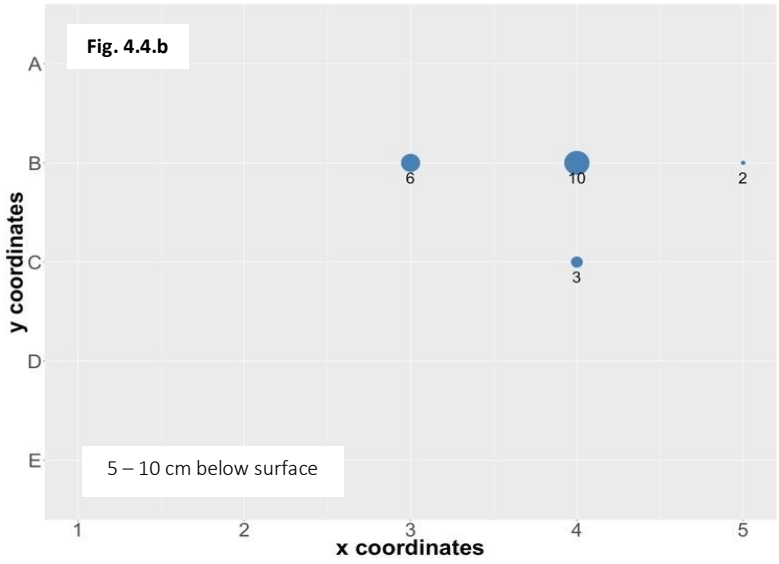
4.3.1 Field experiment

This section provides an account of three experiments that were conducted in the field.

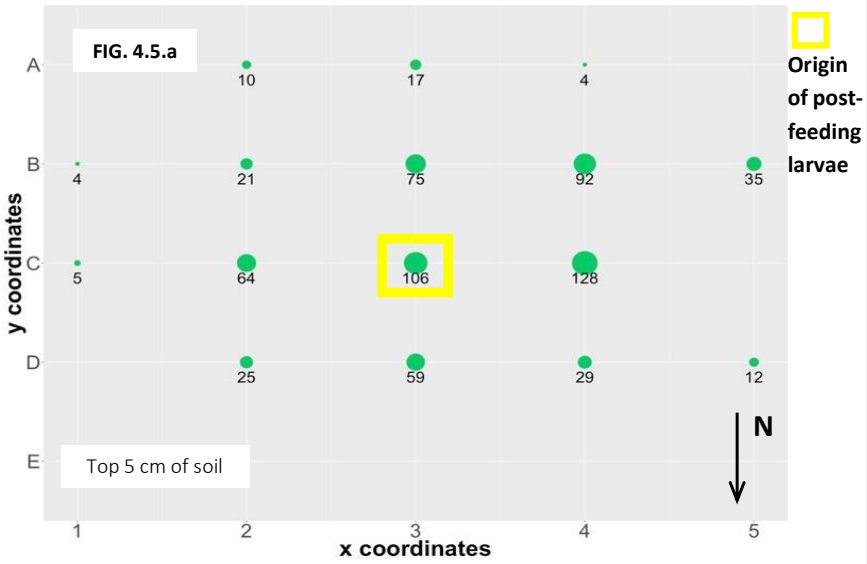


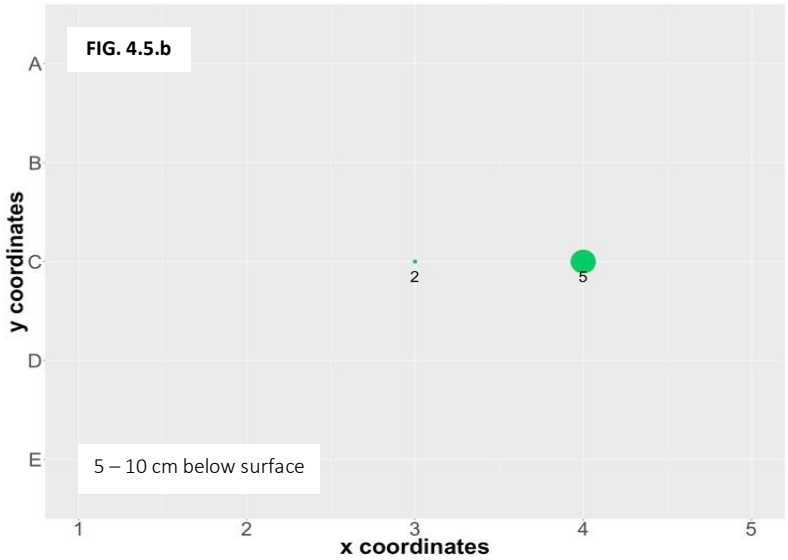
Figures 4.3.a and 4.3.b: The results of the first field experiment are shown. The number of *C. vicina* puparia recovered from each 20 cm x 20 cm section of soil are shown. Figure 4.3.a represents the top 5 cm of sampled soil and Figure 4.3.b represents 5 - 10 cm depth of soil sampled. The entire grid represents a 1 m^2 area. The size of the circle in each square is proportional to the number of puparia recovered from each section (also shown). The x and y coordinates represent a 0.5 m grid around the origin, where the larvae were released at C,3 and the origin of the post-feeding larvae is highlighted by a yellow square. n = 576. 600 larvae were introduced and therefore 96 % of puparia were recovered. North is shown.





Figures 4.4.a and 4.4.b: The results of the second field experiment are shown. The number of *C. vicina* puparia recovered from each 20 cm x 20 cm section of soil are shown. Figure 4.4.a represents the top 5 cm of sampled soil and Figure 4.4.b represents 5 - 10 cm depth of soil sampled. The entire grid represents a 1 m^2 area. The size of the circle in each square is proportional to the number of puparia recovered from each section (also shown). The x and y coordinates represent a 0.5 m grid around the origin, where the larvae were released at C,3 and the origin of the post-feeding larvae is highlighted by a yellow square. n = 191. 200 larvae were introduced and therefore, 96 % of puparia were recovered. North is shown.





Figures 4.5.a and 4.5.b: The results of the third field experiment are shown. The number of *C. vicina* puparia recovered from each 20 cm x 20 cm section of soil are shown. Figure 4.5.a represents the top 5 cm of sampled soil and Figure 4.5.b represents 5 - 10 cm depth of soil sampled. The entire grid represents a 1 m^2 area. The size of the circle in each square is proportional to the number of puparia recovered from each section (also shown). The x and y coordinates represent a 0.5 m grid around the origin, where the larvae were released at C,3 and the origin of the post-feeding larvae is highlighted by a yellow square. n = 693. 700 larvae were introduced and therefore, 99 % of puparia were recovered. North is shown.

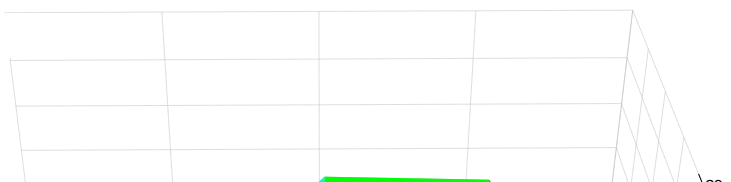
The results of the first field experimental run show limited vertical dispersal, with 82 % of puparia recovered from the top 5 cm of the soil (Figure 4.3.a), with no puparia recovered from deeper than 10 cm. Horizontal dispersal was also limited, with all puparia recovered from within 40 cm of the original coordinate where the post-feeding larvae were placed. With regards to the general distribution of the puparia, there appears to be a general shift West of the origin (Figure 4.3.b).

Again, the results of the second field experimental run show limited vertical dispersal, with 89 % of puparia recovered from the top 5 cm of the soil (Figure 4.4.a), with no puparia recovered from deeper than 10 cm. Horizontal dispersal was also limited, with all of the puparia recovered within 40 cm of the origin. The general distribution of the puparia was shifted West of the origin, slightly more than in the first experimental run and again this is also seen when examining the results of 5 – 10 cm below the surface (Figure 4.4.b).

The results of the third field experimental show even less vertical dispersal, with 99 % of puparia recovered from the top 5 cm of the soil (Figure 4.5.a), with no puparia recovered from deeper than 10 cm. Again, horizontal dispersal was limited, with all puparia recovered within 40 cm of the origin. The general distribution however, was not shifted so much to the West of the origin, rather the distribution appears to be more evenly centred about the origin. However, the numbers of puparia recovered from row 4 were much higher than those in row 2, so there is still a clear West bias.

For all field experimental runs the vast majority of puparia were recovered within the top 5 cm of the soil (Figures 4.3.a, 4.4.a and 4.5.a.). Where more than five puparia were recovered from 5 - 10 cm of soil, the next 5 cm depth were sampled, but in none of the experiments were puparia recovered from > 10 cm deep.

Figures 4.6.a-c show the three field experimental runs in a 3D graph, this representation allows the '3D', bell-shaped, normal distribution to be visualised more clearly. N.B. in graphs 4.6.a-c the frequency data has been converted to percentage, to allow direct comparison of the shape of the figures. The distribution of the puparia recovered is not random, the distribution of the results appears to be a normal ('3D') distribution about the origin, for all three experiments (Figures 4.3.a, 4.4.a and 4.5.a). To clarify, the '3D' distribution refers to the Figures, not the results themselves; i.e. the figures show a '3D' distribution where the x axis represents the x coordinates of the sampled area, the y axis represents the y coordinates of the sampled area and the z axis represents the percentage of puparia recovered (Figures 4.6.a, 4.6.b and 4.6.c). Therefore, the



null and first alternative hypothesis can both be rejected and the second alternative hypothesis accepted as the distribution of the puparia recovered was in a '3D' normal distribution.

Fig. 4.6.a **First field experimental run**

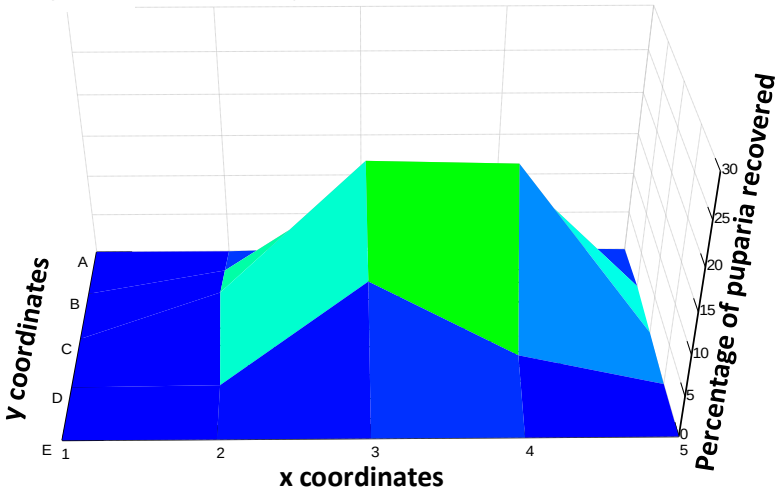


Fig. 4.6.b **Second field experimental run**

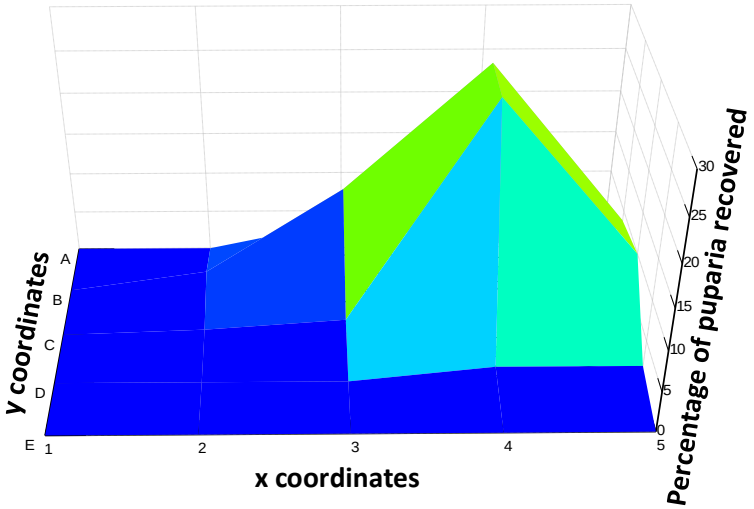
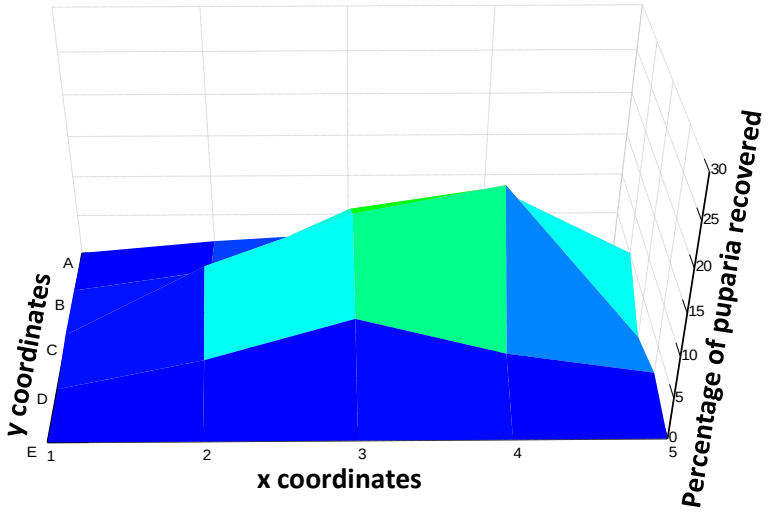


Fig. 4.6.c **Third field experimental run**



Figures 4.6.a-c: Surface grid plots of the three field experiments. The entire grid represents a 1 m^2 area. The x and y axis represent the coordinates of the sampled area, where the larvae were released at C,3. The colour of each grid square represents the number of puparia recovered from it, where the warmer the colour the more puparia recovered, i.e.: dark blue = no puparia, turquoise = a medium amount of puparia and green = the most puparia. The height of each square on the z axis shows the number of puparia recovered from that square. For the first experiment n = 576, for the second n = 191 and for the third experiment n = 693.

4.3.2 Temperature and humidity data

Fig. 4.7.a

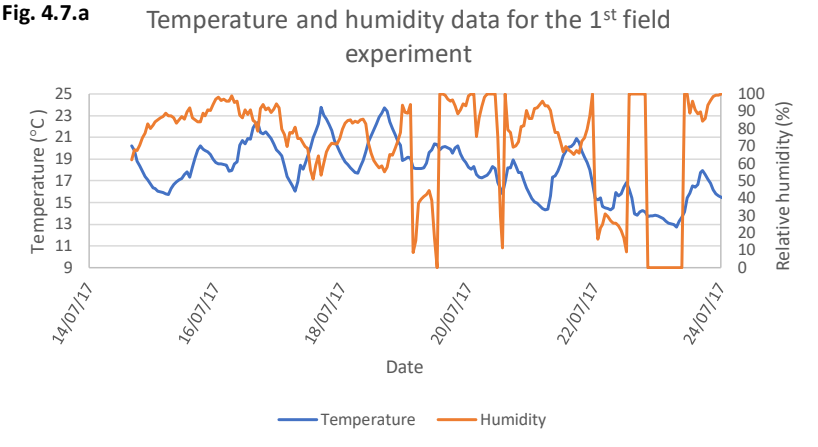
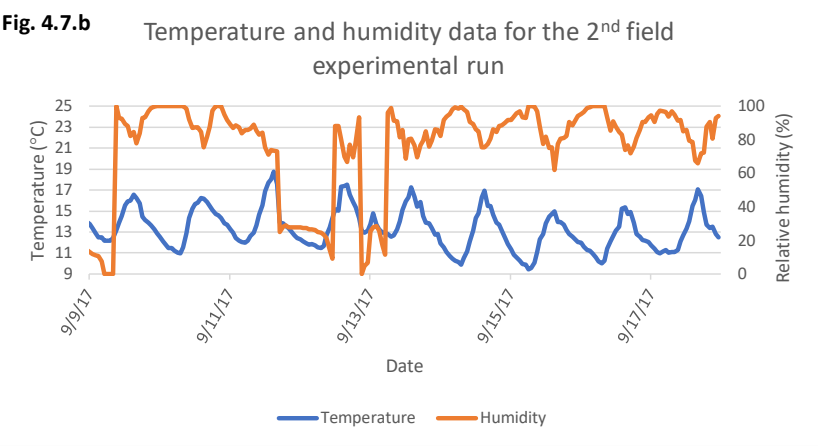


Fig. 4.7.b



Figures 4.7.a and 4.7.b: The temperature and humidity data during the 10 day period of the first and second field experimental runs. Readings were recorded hourly on the hour (Section 4.2.1). No graph is shown for the third experimental run, as the data logger was incorrectly calibrated for this run and the data was therefore not used.
N.B. Clearly there are instances where the dramatic humidity changes are an artefact and a product of a problem with the datalogging device (i.e. 19/07/17, 21/07/17, 22/07/17, 23/07/17, 09/09/17, 12/09/17 and 13/09/17). The data shown from 15/07/17 to 19/07/17 and 14/09/17 to 18/09/17 are reasonable representations of the true humidity.

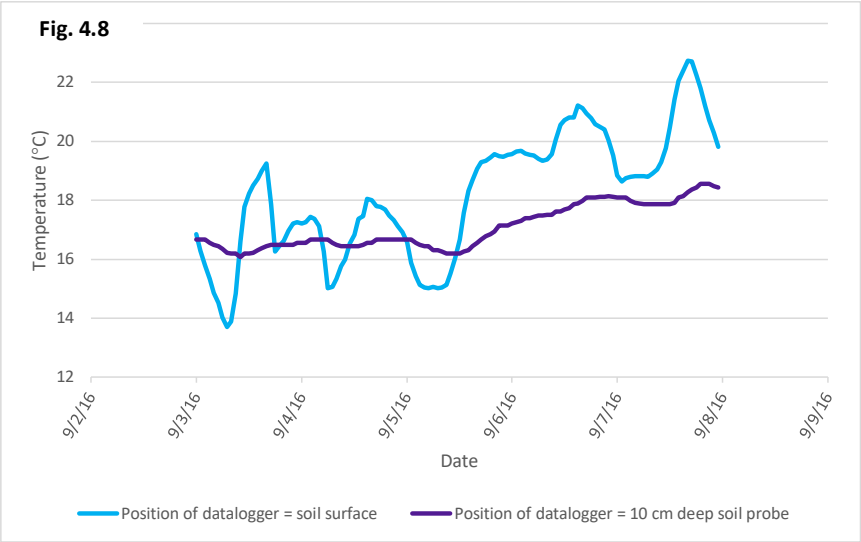


Figure 4.8: Temperature data taken from the Wildlife Garden at the Natural History Museum from the 2nd of September 2016 to the 9th of September 2016, courtesy of Martin Hall (unpublished data). Readings were recorded hourly on the hour using Tinytag Plus 2® Gemini Data Loggers. Temperature was recorded from two data loggers, one placed on the soil as in the field experiments (Section 4.2) and one using a 10 cm probe in the soil.

Figure 4.8 shows the close relationship between the mean soil surface temperature and 10 cm deep in the soil, 18 °C and 17 °C respectively. The temperature recorded on the surface of the soil fluctuates between 14 and 21 °C, whereas the 10 cm deep soil temperature is much more stable, gradually increasing from 16 to 18 °C.

4.4 Discussion

Examination of the temperature data in Figures 4.7.a and 4.7.b shows some similarities. During the first field experimental run the temperature ranged from 13 to 24 °C, with a mean of 18 °C and the second experimental run ranged from 9 to 18 °C, with a mean of 14 °C. The range of the temperature for each run was similar, 11 and 9 °C respectively and the difference between the mean of each was only 4 °C. This is fairly similar, especially where the soil temperature is concerned. When there are fluctuations in the ambient temperature, the soil temperature remains more constant (Figure 4.8 [Fierer *et al.,* 2003]). Post-feeding larvae burrow directly into

the dispersal substrate prior to horizontal or further vertical dispersal (Section 3.4; personal observations; Gomes *et al.,* 2006) and thus the soil temperature directly affects the behaviour and development of the post-feeding larvae more so than the ambient temperature. Considering the mean difference of 4°C in ambient temperature and the relationship between ambient and soil temperature, it can be assumed that the soil temperature during the experimental runs was quite similar and thus unlikely to have had an effect on the behaviour or development of the larvae in the first two experimental runs. More importantly, Figure 4.8 shows how much less variation there is in the soil temperature compared with the ambient temperature. Unfortunately, there was no temperature or humidity data available for the third field experimental run, directly from the Wildlife Garden. Temperature records from the local weather station were compared to the data recorded during this study for the first two experimental runs to determine the level of comparability between the temperatures recorded at the two separate locations. During the first experimental run the local weather station recorded a range of 9 to 24 °C, with a mean of 17 °C (AccuWeather Forecast 2017 [Readings taken from London City Airport]). The minimum temperature recorded by the weather station was lower than was recorded during the study (13 °C), but the maximum (24 °C) and means (18 °C) were very similar. For the second run, the local weather station recorded a range of 6 to 18 °C, with a mean of 13 °C (AccuWeather Forecast 2017 [Readings taken from London City Airport]). Again, the minimum temperature recorded by the weather station was slightly lower than was recorded during the study (9 °C), but the maximum (18 °C) and mean (14 °C) temperatures were very similar. This corroboration of the temperature data from the weather station with the temperatures recorded during the study suggest that the records are very similar and can therefore be appropriately used to estimate the temperature data for the third experimental run. During the third experimental run, the local weather station recorded a temperature range of 8 to 21 °C, with a mean of 11 °C (13th to the 23rd October 2017) (AccuWeather Forecast 2017 [Readings taken from London City Airport]). The temperature range is less than the other two experimental runs and the mean temperature is between the other means. Therefore, again, temperature is unlikely to have had an effect on any differences in behaviour or development between all three experimental runs.

Ambient relative humidity has little effect on the dispersing post-feeding larvae as they burrowed into the soil as soon as they were released. The soil moisture content is more stable than the relative humidity and is more closely related to soil temperature (Tromp-Van Meerveld and Mcdonnell, 2006). Thus, any fluctuations in relative humidity that were recorded are unlikely to have had an effect of the larvae (Figures 4.7.a and 4.7.b).

The general results of the field experiments were as expected. The limited vertical dispersal recorded during this experiment, compared with the results of vertical dispersal (Section 3.3.3) laboratory experiments, was anticipated (Section 4.1). The null hypothesis for vertical dispersal can be rejected and the alternative accepted, the majority of puparia were recovered from the top 5 cm of the substrate, supporting the results of the simultaneous horizontal and vertical experiments (Section 3.3.4). During the vertical dispersal laboratory experiments the dispersing larvae were given the option of vertical dispersal only and therefore, naturally the results of the experimental design are exaggerated and show primarily the ability of larvae to disperse vertically, not the likelihood. The field experiment results most closely resembled the simultaneous horizontal and vertical experiment (Section 3.3.4), where the post-feeding larvae were given the choice to move vertically and/or horizontally. There does appear to be, both in the laboratory and field experiments, a preference of the post-feeding larvae to disperse further horizontally than vertically, even though they are clearly capable of dispersing a considerable vertical distance (Section 3.3.3), which is also reported in the literature (Godoy *et al.*, 1995; Cammack *et al.*, 2010).

The horizontal dispersal recorded during this experiment was also expected. The majority of puparia were also recovered from within 20 cm of the point of origin: 80 % for the first experiment, 67 % for the second and 86 % for the third (Figures 4.3.a, 4.4.a and 4.5.a). Therefore, the null hypothesis can be accepted and the alternative rejected as in fact all of the puparia were recovered from within 4 m of the origin.

The puparia were not recovered in an even distribution or a random distribution from any of the experimental runs. The pattern of the puparia recovered was in approximately a '3D' normal distribution and therefore the second alternative hypothesis can be accepted. Perhaps the areas sampled were too big (20 x 20 cm) to recognise any more subtle patterns, such as aggregations. In fact, when searching for the puparia by hand, they were often found in small groups. It is also important to note that the dispersal substrate in the Wildlife Garden was not homogenous, as in the laboratory experiments, and this can alter the dispersal patterns of post-feeding larvae (Cammack *et al.*, 2010). However, a relatively clear patch of topsoil was chosen and few roots and rocks were encountered during sampling (Figure 4.1.a). There was a slight Westerly bias of the puparia recovered about the origin seen for all the experimental runs, but the bias was less pronounced in the third run. As far as the author is aware there was no biotic or abiotic factor that differed during the third run compared to the other two, other than the location of the run in the Garden (Figure 4.2.b). The third run was located approximately 3 m from the location of the second run and there appeared to be no obvious differences in the location with regards to

the environment (e.g. tree canopy cover, soil type, location of large plants/ trees, distance from the wall of the garden etc). There was also no significant difference in the temperature or humidity recorded during the runs. Therefore, the author concludes that the slight difference in location of the recovered puparia in the third experimental run compared to the first two runs was either due to chance or to a factor unknown to the author and thus not measured. Moreover, the difference accounted for a 20 cm shift in the location of the recovered puparia and thus the variation in the results was very small. As far as the author is aware, there was also no reason for the slight Westerly bias seen in all of the runs. The larvae were released at noon for each run and therefore the position of the sun should not have affected the directionality of dispersal, especially as the larvae immediately burrowed into the substrate. There was a footpath on the Easterly side of the experimental location and perhaps this affected the dispersing larvae (Figure 4.2.b).

These field experiments have highlighted the importance of field studies to enhance laboratory studies. It is easy to become obsessed with the minutia of experimental results under controlled conditions, but often in the field these small details are hidden due to the increased variability of the conditions.

In conclusion, the results of this Section support the results of the laboratory experiments conducted in the Sections reported thus far. Similar and expected results were found in the field, when compared with the laboratory studies.

Chapter 5: Experiments conducted using a servosphere

5.1 Introduction

The experiments conducted in this Chapter used an experimental apparatus called a servosphere, originally designed by Ernst Kramer in 1976 (Kramer, 1976), to enable the observation of the walking behaviour of podous insects. The apparatus consists of a sphere, at the apex of which an insect is placed; the sphere turns to compensate for the insect's movement, such that it allows the unimpeded movement of a single insect in any direction (Figure 5.1; Section 5.2). The displacements of the sphere generated by the insect's movement, were measured at an interval of 0.1 s with an accuracy of 0.1 mm. Through this mechanism the walking behaviour of an insect can be studied and any behavioural changes to external stimuli recorded. The insects that have been studied using this apparatus include Coleoptera (McMahon and Guerin, 2000; Otálora-Luna and Dickens, 2011), Hemiptera (Taneja and Guerin, 1995; Otalora-Luna *et al*., 2004), Blattodea (Bell and Kramer, 1980), Lepidoptera (Sakuma, 2002) and other arthropods, such as mites (Rickli *et al*., 1992).

The servosphere allows the walking behaviour of the insect to be studied and any behavioural changes to external stimuli recorded. Previous studies have examined insect responses, such as walking towards, away from or stopping, as a result of different stimuli, such as volatiles and different wavelengths of light (Kramer, 1992; Arnold *et al.,* 2016). Kramer (1976) used the servosphere to examine walking honey bees in different odour fields and the servosphere removed the 'edge effect', which had not been possible with previous experimental setups. Van Tilborg *et al.* (2004) used a servosphere to examine the predatory mite *Phytoseiulus persimilis* and its response to different volatiles. The servosphere allowed the use of constant air speeds and constant concentrations of the volatile that had been impossible with previous experimental setups (van Tilborg *et al.*, 2004). Other studies that examined the response of different species of insect to different volatiles include: Taneja and Guerin (1995), who examined the responses of the triatomine bugs *Rhodnius prolixus* and *Triatoma infestans* and McMahon and Guerin (2000), who examined the responses of the tropical bont tick, *Amblyomma variegatum*. Studies of the response of different species of insect to wavelengths of light include: Preiss and Kramer (1984), who examined the phototactic (active movement towards or away from light) behaviour of the walking gypsy moth *Lymantria dispar*, Otálora-Luna and Dickens (2011), who studied the response of the Colorado potato beetle, *Leptinotarsa decemlineata* and Otálora-Luna *et al.*

(2013), who examined the response of the tropical root weevil *Diaprepes abbreviatus* to different wavelengths of light.

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As far as the author is aware, the servosphere has only been used to study the behaviour of podous insects and thus the use of it to study an apodous larva is novel. The experiments conducted using the servosphere were carried out to examine multiple factors of post-feeding larval dispersal under highly controlled conditions. *Calliphora vicina* was the main species used because the previous chapters have focused on this species and therefore most of the experimental data pertains to *C. vicina*. However, some experiments were conducted with *P. terraenovae* as this species is known to remain close to the body (Erzinçlioğlu, 1996; Arnott and Turner, 2008; Byrd and Castner, 2009) and was therefore identified as an appropriate species to provide data for comparison with *C. vicina*.

The main factors of post-feeding larval dispersal that were studied included speed and total distance dispersed. It has been suggested that dispersal speed increases as a function of larval length (Charabidze *et al.*, 2008). Therefore, as fully developed *C. vicina* larvae (20 - 22 mm) are longer than *P. terraenovae larvae* (18 - 20 mm) (Smith, 1986), the speed of the former should be greater than that of the latter. It was not possible to record the speed of larval dispersal in the substrates tested in Chapter 3, as the initial larval burial obscured their subsequent movements. Moreover, as cardboard was instantly placed on top of all experimental apparatuses, to ensure light was not a variable, even observing larval dispersal in the bare gutter (no substrate) was not possible. Thus, the servosphere allowed the only recording of post-feeding larval speed in this study.

Not only should there be a difference in the larval speed of *C. vicina* compared to that of *P. terraenovae* if larval length is related to speed, but there should be an overall decreasing speed over time. This latter theory has, as far as the author is aware, not been tested previously. Pupariation is initiated by the production of moulting hormones during the post-feeding larval stage and one hormone known to be involved in this process is ecdysone (Thummel, 1996; Beckstead, Lam and Thummel, 2005). The concentration of the hormone ecdysone, which is supressed during the larval stages, increases prior to pupariation (Thummel, 1996; Beckstead, Lam and Thummel, 2005). Logically therefore, there should be a gradual decrease in the speed of post-feeding larvae, as their concentration of moulting hormones increases as they near pupariation.

Experiments examined the distance travelled and changes in speed during one hour by *C. vicina*, the changes in speed and distance dispersed, by *C. vicina*, every day for four days and a comparison of the speeds and overall distance dispersed between *C. vicina* with *P. terraenovae*. A final experiment was conducted to examine post-feeding larval response to light. Calliphorid larvae possess light receptors (ocelli) which enable them to detect light (Byrd and Castner, 2009; Hinnemann *et al.*, 2010). It is well known that post-feeding larvae react negatively to the stimulus of light, as they are in search of a suitable pupariation site and this is often within the dispersal substrate itself (i.e. somewhere dark) (Benecke, 2005; Hinnemann *et al.*, 2010). This is clearly observable in many situations as the post-feeding larvae disperse away from sources of light and burrow into the pupariation medium as soon as they are able (Chapter 3 and 4). This phenomenon, however, has not been studied under highly controlled conditions such as are possible with the servosphere.

5.1.1 Hypotheses for the experiments conducted using a servosphere

The first experiment conducted examined the changes in the larval speed of *C. vicina* over one hour. The hypotheses for this experiment were as follows:

H_0: There would be no change in average speed over time.

H_1: There would be a decrease in speed over time.

H_2: There would be an increase in speed over time.

The second experiment examined the differences between the speed of *C. vicina* and *P. terraenovae* over a four day period and the hypotheses for this experiment was the same as for the first experiment:

H_0: There would be no change in average speed over time.

H_1: There would be a decrease in speed over time as the larva neared pupariation.

H_2: There would be an increase in speed over time

An additional set of hypotheses examined the differences between the two species:

H_0: There would be no difference in the average speed or overall track length between *C. vicina* and *P. terraenovae*.

H_1: The average speed or overall track length of *C. vicina* will be more than that of *P. terraenovae.*

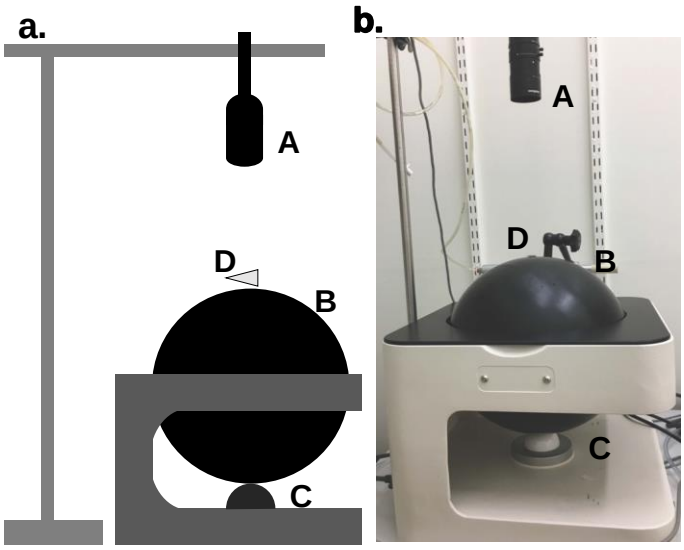
The third and final experiment examined the response of *C. vicina* larva to light. The hypotheses for these experiments were:

H_0: The larvae would show no response to the light source.
H_1: The larvae would show a negative tactic response to the light source.
H_2: The larvae would show a positive tactic response to the light source.

5.2 Methodology

5.2.1 Servosphere laboratory experiments

All experiments in this Chapter were conducted using a Syntech TrackSphere LC-300 (Syntech, Hilversum, Netherlands) servosphere. The servosphere apparatus enabled one larva to be examined per experiment in a temperature and humidity controlled setting (Figure 5.1.a and 5.1.b). The servosphere apparatus consisted of the servosphere, a CMOS (complementary metal-oxide semiconductor) camera and a control unit (Figure 5.1.a and 5.1.b). A larva was placed at the apex of the sphere, the camera visually tracked the insect and the servosphere moved, via servomotors, such that the larva's position was maintained at the apex of the sphere. In this way the servosphere allowed unimpeded larval movement in any direction, whilst maintaining the larva's position at the apex of the sphere. The larval tracks were recorded using TrackSphere 3.1 (Syntech, Hilversum, Netherlands) software. The software provided both raw and partially processed data that included the raw coordinates of the larval movements, diagrams of the larval tracks, average speeds and track lengths. The room was temperature controlled and set to 23°C. A factory calibrated Tinytag Plus 2® data logger was placed in the room to record the temperature and humidity. Lux light levels were measured daily using a Heavy Duty Lightmeter (HD450) with the sensor aimed vertically upwards or at a 45 ° angle in relation to the vertical and horizontal plane of the room (Figure 5.1.a and 5.1.b).



Figures 5.1.a and 5.1.b: Schematic of the servosphere apparatus (a.) and the servosphere in situ (b.). The apparatus consists of a CMOS camera (A), the servosphere sphere (B) the servomotor (C) and the position of the larva is shown at D, with the arrow indicating the direction of the larval placement.

Three experiments were conducted using the servosphere apparatus, the first with three runs, the second with ten and the third with three. The larvae used in the servosphere experiments were all in the post-feeding stage and reared using the methods described in Section 2.2.1. All larvae were placed on the sphere in the direction indicated in Figure 5.1.a, with the head towards the left from the experimenter's perspective. A larva was placed at the apex of the sphere and given one minute to acclimate to moving on the sphere; the larva was then gently moved with forceps to ensure that it began each experiment facing in the correct direction, i.e. facing left. Between each experimental run the sphere was cleaned by wiping it entirely with 70 % ethanol solution. Initial studies showed that dry larva rolled directly off the sphere, therefore, to ensure that the larvae continued to move on the sphere unimpeded each larva was kept moist by adding a drop of deionised water directly onto the larva; this ensured the larva retained traction on the sphere.

5.2.2 *Larval speed changes during one hour experiment*

The first experiment was conducted using the servosphere apparatus to observe the behaviour of post-feeding *C. vicina* larvae over time. The experiment specifically examined the changes in speed of each larva over one hour. This experiment was conducted on the 6th of November 2017.

Three replicates were carried out. One larva was used per replicate, i.e. three larvae in total were used, all at the same stage of development. The average speed (mm/s) of each larva was recorded per minute for one hour.

5.2.3 *Comparison of Calliphora vicina and Protophormia terraenovae on a servosphere*

The second experiment conducted using the servosphere apparatus examined the differences exhibited by two different species of blow fly: *C. vicina* and *P. terraenovae*. The experiment studied differences in speed and differences in the total distance dispersed, over a period of four days. Each day the experiment was repeated with ten specimens of *C. vicina* and ten specimens of *P. terraenovae*. The same 20 specimens were used each day, unless they had pupariated. Each run lasted for five minutes. The details of the experiment are summarised in Table 5.1.

Table 5.1: Summary of some of the details of the second servosphere experiment. The Table includes the day of the experimental run, the number of specimens used in each run, the duration of the run and the species used. The experiments were conducted from the 6th to the 9th of November 2017,

Day	Number of specimens	Run duration (minutes)	Species
1	10	5	*C. vicina*
1	10	5	*P. terraenovae*
2	10	5	*C. vicina*
2	10	5	*P. terraenovae*
3	10	5	*C. vicina*
3	10	5	*P. terraenovae*
4	10	5	*C. vicina*
4	10	5	*P. terraenovae*

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5.2.4 Response of Calliphora vicina to light

The fourth experiment using the servosphere apparatus was conducted to determine whether the larvae exhibited any response to light, for example the expected negative phototactic response (physical movement away from a light source) (Miller, 1929; Benecke, 2005; Hinnemann *et al.*, 2010) (Section 1.6.9). The negative phototactic response of blow fly larvae is well known and cited (Hinnemann *et al.*, 2010), as it is easily observed, however to date this response has not been examined under highly controlled conditions such that the negative response to light can be conclusively isolated.

Post-feeding *C. vicina* larvae were used and the experiment was repeated three times. During each experiment there were four runs. A different larva was used for each run and each experiment, such that 12 larvae were used in total. During this experiment each run began with the larvae facing left (Figure 5.1.a). All of the lights and other light sources (lamp) in the experimental room were turned off such that lux readings of 0 were taken throughout the room (there was still light emitting from the computer screen, but as the lux readings were 0, it was determined that this would not interfere with the experiment). A larva was placed at the apex of the sphere and an external lamp (iPhone torch) was then shone (Figure 5.2) at the larvae from directly above (the lamp was hand held). Once the larval movement was recognised by the camera, the position of the light was moved, by hand, so that it shone at 45° to the line of sight of the camera and the larva (Figure 5.2). The lamp was then moved for each run in four different directions (Figure 5.3 and Table 5.2). Lux light levels of the lamp were measured at the start of each experimental run using a data logging Heavy Duty Lightmeter (HD450) with the sensor aimed vertically upwards, the mean lux level produced was 3000 lux, with a range of 2250 to 3460 lux.

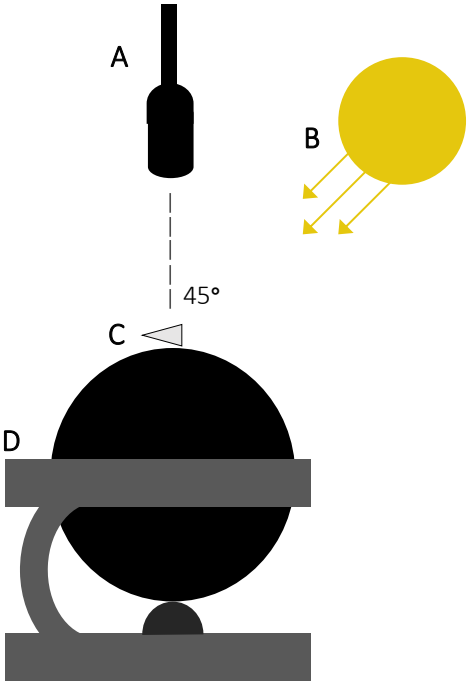

Figure 5.2: Schematic of the servosphere apparatus with the external light source shown. The apparatus consists of a CMOS camera (A), the light source (B), the servosphere sphere (D) and the position of the larva is shown at C. The direction of light shown is 'West' (Figure 5.3) and the light is positioned such that the light hits the larva at 45° to the larva and camera.

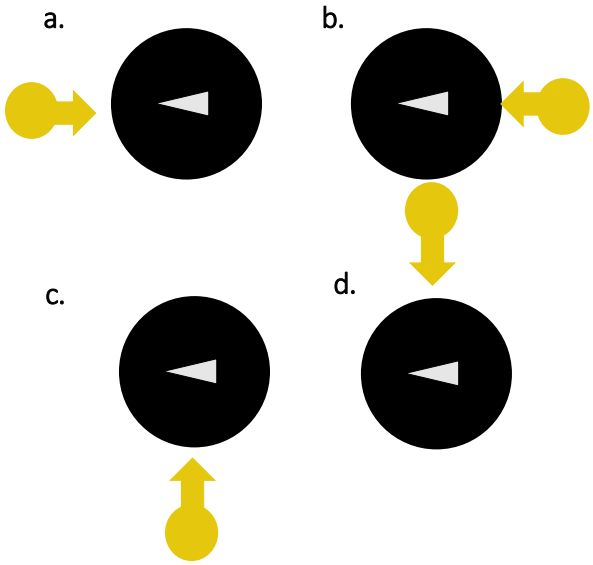

Figure 5.3: Schematic showing the sphere from above with a larva in the starting position. The direction of the light source is also shown: a. the direction of light shining towards 'East', b. the direction of light shining towards 'West', c. the direction of light shining towards 'North' and d. the direction of light shining towards 'South'. All of the larvae began each run facing 'left' as during the rest of the experiments (Section 5.2.1 and 5.2.2).

Table 5.2: Summary of fourth servosphere experiment, using *Calliphora vicina*. More information about the direction of the light source is provided in Figure 5.3. The experiments were conducted on the 10th of November 2017.

Direction of light source	Run duration (minutes)
East	3
West	3
North	3
South	3
East	3
West	3
North	3
South	3
East	3
West	3
North	3
South	3

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5.3 Results

5.3.1 Larval speed changes during one hour experiment

The results of the larval speed changes during one hour experiment are summarised in Figure 5.4.

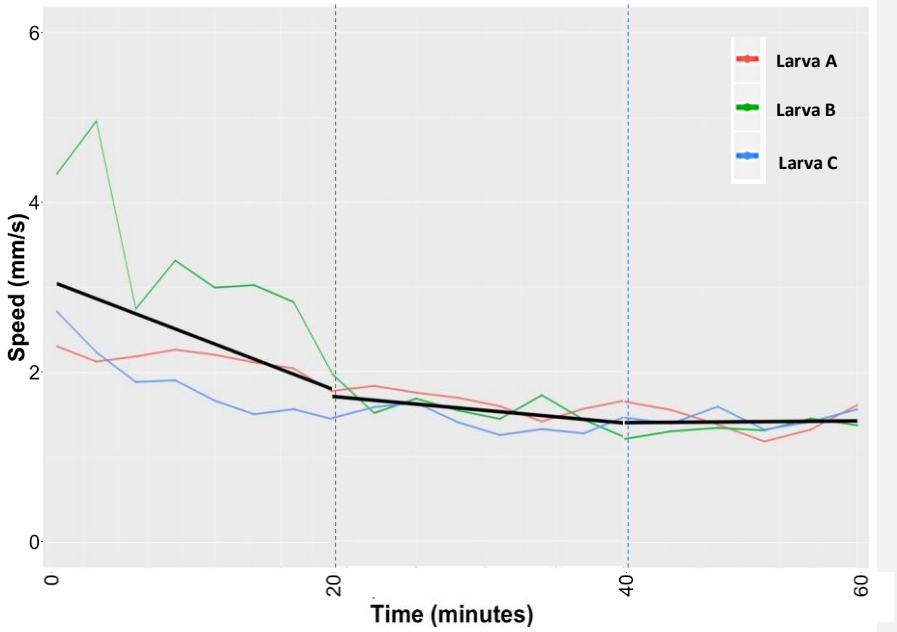


Figure 5.4: The change in the larval speed (mm/s) of each *Calliphora vicina* larva: A, B and C over one hour. The average speed was recorded every three minutes. Three linear regression lines of the means at each time point are fitted onto the graph (black lines), for 0 to 20 minutes, 20 to 40 minutes and 40 to 60 minutes.

The average speed, of each larva, over one hour decreased. The speed of each larva fluctuated slightly over time, but the general trend for the speed of all three larvae was decreasing. The speed of larva A and C decreased at a fairly constant rate, whereas the speed of larva B began much higher than the other two and increased sharply until 5 minutes after the experiment commenced, where the rate of deceleration decreased such that the larval speed was similar to that of the other two larvae.

When the time periods of 0 to 20 minutes, 20 to 40 minutes and 40 to 60 minutes are examined separately the trendlines of each time period are different, with the gradient of each line becoming less steep over time: the gradient of the trendline for the first 20 minutes is -0.056, the gradient of the trendline for the second 20 minutes is -0.018 and the gradient of the trendline for the third 20 minutes is 0.0013. The larval speed of all three larvae was decreasing over time, till the speed was almost constant (last 20 minutes). Therefore, the primary alternative hypothesis can be accepted and both the null and secondary alternative hypotheses can be rejected, as larval speed decreased over time.

This experiment was also initially conducted to determine after how long the larva would cease dispersal, after continual movement. However, this experiment was concluded after 60 mins when the cessation point had not been reached. Larva A dispersed a total of 7740 mm in one hour, larva B dispersed 7681 mm in an hour and larva C dispersed 5796 mm in one hour.

5.3.2 Comparison of Calliphora vicina and Protophormia terraenovae dispersal on a servosphere

Ten *Calliphora vicina* and ten *Protophormia terraenovae* larvae, all of which had just entered the post-feeding stage, were used during this experiment to determine whether there were any differences in the average speed per run over time exhibited by the larvae and if there was a difference in the average speed exhibited by each species. The overall track length of each run was also examined. This experiment was different from the previous experiment (Section 5.3.1) as it considers the differences between species, the experiment lasted four days, and also each larva was not isolated between runs and therefore cannot be compared as such (i.e. the larvae were unidentifiable and therefore the data was pooled). Each larva was run for 5 minutes once a day, for four days. Between runs the larvae were returned to a 1.3 litre Drennan Maggibox with the other larvae, of the same species, that were used during this experiment and held at 3 °C.

The results of the experiment are summarised in Figures 5.5.a and 5.5.b. Again, it was thought that there would be a decrease in the average speed over time, and thus the average track length, as the larvae neared pupariation. Additionally, it was thought that the speeds and overall track length of *P. terraenovae* would be slightly less than that of *C. vicina*, because of the smaller average size of the post-feeding third instar *P. terraenovae* larva compared to that of *C. vicina*.

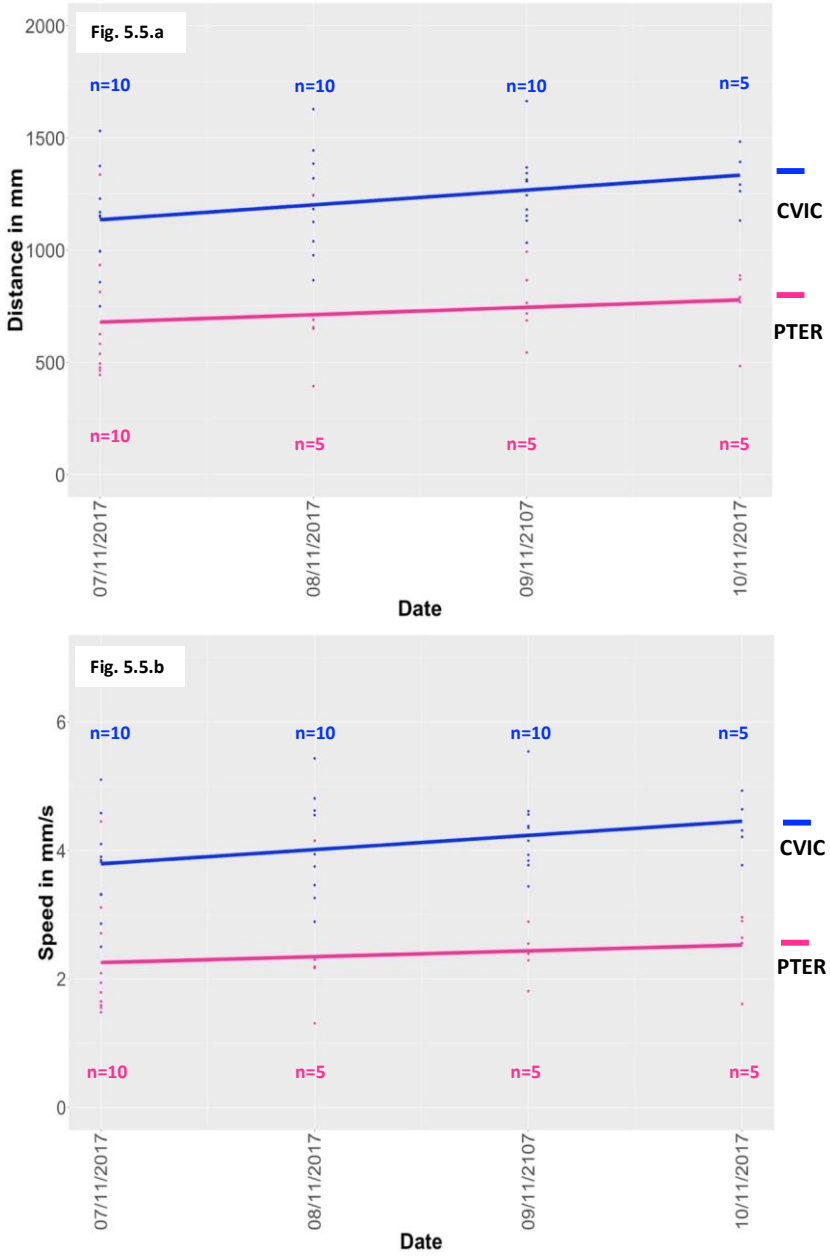


Figure 5.5.a: The overall track length of *C. vicina* (CVIC) and *P. terraenovae* (PTER) over four days. **Figure 5.5.b:** the average speed of *C. vicina* and *P. terraenovae* over four days. A linear regression line is fit onto each graph and shown in either blue for *C. vicina* or pink for *P. terraenovae*. The length of each run was 5 minutes. The value of n decreases over time as some of the larvae had pupated (the blue values of n refer to *C. vicina* and the pink to *P. terraenovae*).

Although there was a large variation in the individual results of this experiment (Figures 5.5.a and 5.5.b), the overall trend, for each species tested, shows a slight increase in speed and overall track length over the four day period (Figure 5.5.a and Figure 5.5.b). As overall track length and speed are very closely related, the following text will discuss the speed only, as all comments also relate to overall track length. A t-test was carried out in RStudio® to determine whether the slopes of the linear regression lines were significantly different to a gradient of 0 and the results are summarised in Table 5.3. As the slope of the linear regression lines for *C. vicina* and *P. terraenovae* are both significantly positive, this shows that the speed of each species is indeed increasing over time (Table 5.3). This result was unexpected for two reasons. Firstly, given that the results of Section 5.3.1 showed that at the initiation of the post-feeding larval dispersal stage larval dispersal speed initially decreased and then remained almost constant after 40 minutes. Given the results of the previous experiment (Section 4.3.1) larval dispersal speed was anticipated remain constant over time, assuming the trend seen in the experiment continued. The results of this experiment show that this is clearly not the case. It was assumed that when the larvae are not being examined they continued to move, however this was not confirmed as the larvae were not continually observed. Ideally each larva would have been run and been observed continually over the four day period, though without more servospheres and operators this was not possible. The second reason why the results of the second experiment (Section 5.3.2) were unanticipated was due to the hypothesis that over time, as each larva neared pupariation, their speed would decrease (Sections 5.1 and 5.1.1). This hypothesis was due to an increase in the concentration of moulting hormones in the larvae prior to pupariation; these hormones are known to be involved in the initiation of pupariation (Thummel, 1996; Beckstead *et al.,* 2005). These results (Sections 5.3.1 and 5.3.2) highlight the complexity of post-feeding larval dispersal and the need for further examination of this complex stage of development (Section 6.1.5). This is discussed in further detail later in this Chapter (Section 5.4.1).

Table 5.3: The results of four t-tests carried out on the experiments outlined in this Section and presented in Figures 5.5.a and 5.5.b, to determine whether the slopes of the linear regression lines were significantly different to a gradient of 0.

Species	Data	Slope	t- value	*p*- value
C. vicina	Average speed	Positive	2.443	0.0201
P. terraenovae	Average speed	Positive	6.203	<0.0001
C. vicina	Track length	Positive	2.456	0.0195
P. terraenovae	Track length	Positive	6.143	<0.0001

The average daily speed of the *C. vicina* larvae was higher than that of *P. terraenovae* (Figure 5.6). A t-test was conducted to confirm this and the results were highly significant: t = 8.92, $p < 0.0001$ (*C. vicina* $\bar{x}$=4.08 mm/s and *P. terraenovae* $\bar{x}$=2.36 mm/s). Moreover, the mean average speed of *C. vicina* in this experiment was $\bar{x}$=4.08 mm/s, which equates to a mean speed of 14 m/hr and the mean average speed of *P. terraenovae* was $\bar{x}$=2.36, which equates to 8.5 m/hr. The higher average speed of *C. vicina* compared to *P. terraenovae* was anticipated due to the average larger size of fully developed *C. vicina* larvae (Section 4.1) and speed has been shown to increase as a function of body length (Charabidze *et al.*, 2008).

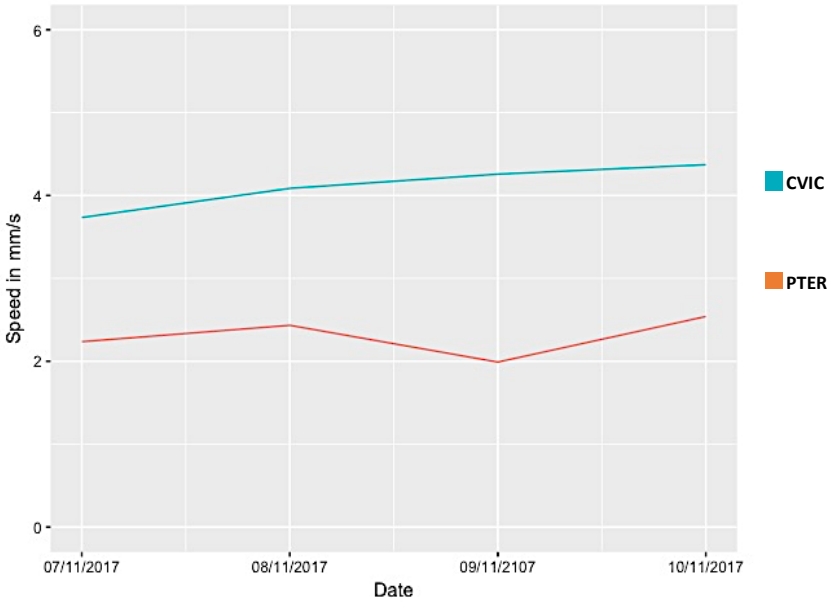
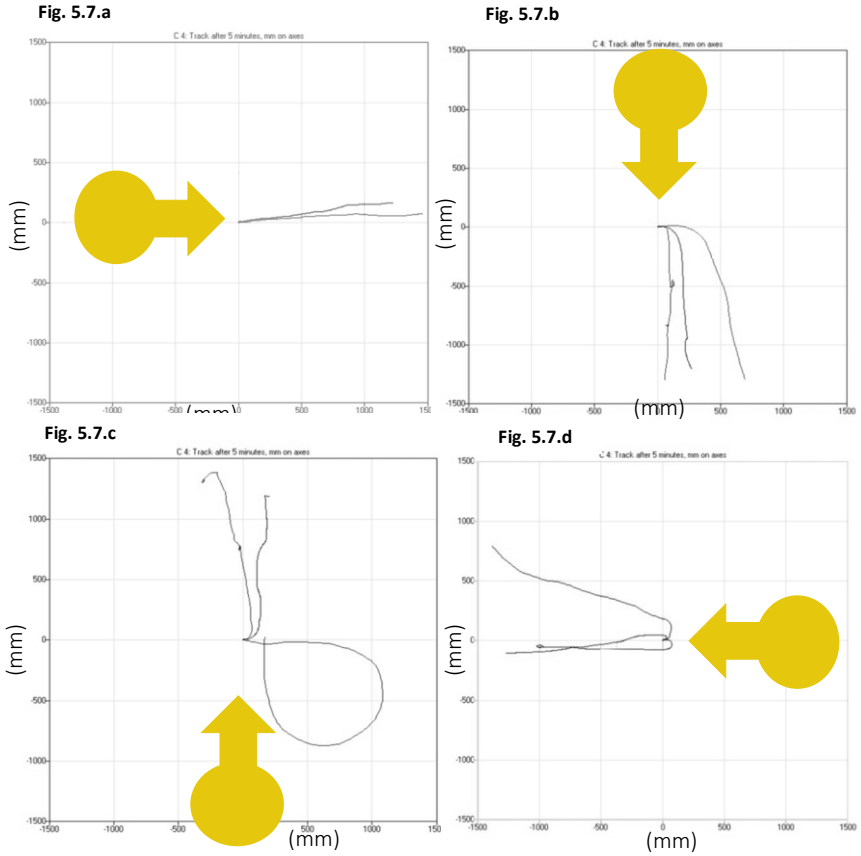


Figure 5.6: The average daily speed of 10 *C. vicina* larvae over four days, compared with that of 10 *P. terraenovae* larvae. The length of each run was 5 minutes.

5.3.3 Response of Calliphora vicina to light

As clearly visible from the tracks of the larvae during this experiment (Figure 5.7.a-d), each larva exhibits a strong negative response to light. Each larva was run for 5 minutes and it is clear that the initial negative larval movement from the light source occurred within 5 seconds and that there was continued larval taxis directly away from the light source. During the first three runs

(Figure 5.7.a) the light source was introduced directly behind each larva, such that the larval negative phototactic behaviour produced a straight track directly away from the light source for each run. The second three experimental runs (Figure 5.7.b) introduced the light source to one side ('South') of each larva (with the larva again starting the run facing right), the negative phototactic response occurred such that each larva turned approximately 45° to their right and continued dispersal away from the light source, producing nearly straight tracks. The same response was seen in the third set of runs (Figure 5.7.c), but in the opposite directions as the light source was introduced to the opposite side ('North') of each larva. One of the larval tracks (Figure 5.7.c) did show an initial movement towards the light source, at a much shallower angle than the other larvae moving away from the light source, however within the 5 minute run the larva had completed a full circular track to then continue in a straight line away from the light source, as the other two larvae had done. The final set of experimental runs introduced the light source directly facing each larva and again, within seconds, each larva turned approximately 180° and continued to produce a straight track directly away from the light source (Figure 5.7.d).



Figures 5.7.a-d: Larval tracks of 12 *C. vicina* larvae. The tracks are shown on a 1500 x 1500 mm grid, where 0,0 was the origin of each larvae. Each larval run began with the larva facing right ('East'). A light source was then shone at 45° towards the larva from four different directions: 'East' (Figure 5.7.a), 'South' (Figure 5.7.b) 'North' (Figure 5.7.c) and 'West' (Figure 5.7.d), as indicated by the yellow circles with directional arrows. The length of each run was 5 minutes.

5.3.4 *Temperature, humidity and lux data*

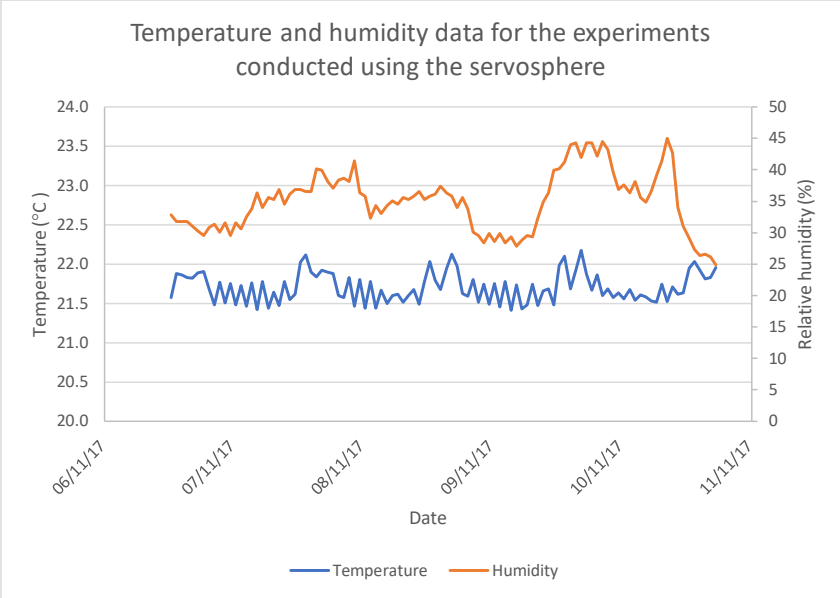


Figure 5.8: The temperature and humidity readings recorded on the Tinytag Plus 2® data logger that was placed in the servosphere experimental room. Readings were taken every 10 minutes.

The temperature and humidity data presented in Figure 5.8 shows how little variance there was in the temperature and humidity over the five days that the experiments were conducted. The temperature ranged from 21.4 °C to 22.2 °C, with a mean of 21.5 °C. Although the room was set at 23 °C and clearly this temperature was not reached, the small variation of the temperatures is important. There was also a low variance in the relative humidity; the humidity varied from 25.0 % to 45.0 %, with a mean of 38.9 %. This data (Figure 5.8) suggests that the temperature and humidity caused none of the variations seen in the results of the experimental runs. There was also a low variance in the light intensity in the room as can be seen in Figure 5.9. Again, any variability seen in the lux readings was so little that it appears unlikely to have influenced the larvae used during any of the experiments outlined in this Chapter.

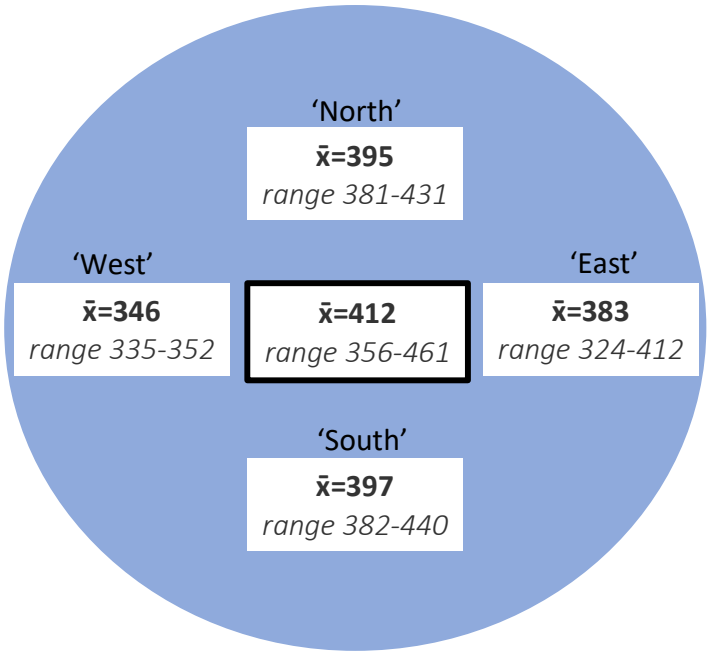


Figure 5.9: Results of the lux readings taken once a day, over a 5 day period. The readings were taken from four different positions: the sensor aimed vertically upwards for the central readings (shown with a black outline to the box) or at 45° to the line of sight of the camera and the horizontal plane at positions 'North', 'East', 'South' and 'West'. The mean lux reading for each position is shown as is the range. All measurements are in lux.

5.4 Discussion

5.4.1 Larval speed changes during one hour and the comparison of Calliphora vicina and Protophormia terraenovae on a servosphere

The servosphere apparatus and set up allowed for the experimentation and analysis of individual calliphorid larvae in a highly controlled setting. The temperature and humidity were controlled, with very little variability (Figure 5.8); temperature was the most important variable to maintain as the speed of larval dispersal is known to increase with temperature (Charabidze *et al.*, 2008). The light throughout the room was also fairly even (Figure 5.9). Although the experiments conducted using the servosphere measured different properties (i.e. speed) than the other laboratory experiments (Chapter 3), the data produced from the experiments in this Chapter was very precise, much more so than was possible during the other experiments conducted in this study. This study was also the first to use a servosphere to examine apodous insects and thus the results outlined in Section 5. 3 are novel.

One of the initial experiments attempted was to observe and record the dispersal capacity of a calliphorid larva (this experiment is not included in the results [Section 5.3] as the experiment was terminated before any data was produced). When larvae naturally disperse into a substrate, they initially burrow into the substrate and therefore their movements from then on are unknown until their final pupariation site (Gomes *et al.*, 2006). It is possible to observe larval movement on a hard surface, for example the bare gutter experiments outlined in Chapter 3, however during these studies the larvae were not observed for long and were quickly covered to ensure that all larvae were exposed to no light. Larvae in storage boxes appear to be continually moving (personal observations), however they are constantly disturbed by neighbouring larvae. However, this seems like an unnecessary energy expenditure that could be better used for the insect's development. Thus, without the disturbance of neighbouring larvae it was hypothesised that when a single larva was run unimpeded on the servosphere that the larva would stop intermittently and finally cease moving. Therefore, the initial experiments using the servosphere were meant to run a larva until alternative behaviour was observed. However, after one hour of a continuous run the *Calliphora vicina* larva had not stopped once; this was repeated twice more and the same observation was made. Therefore, the plan to watch an individual larva for an unlimited time was concluded after 60 mins when the cessation point had not been reached; with just a single servosphere available, the priority was directed to other experiments. Moreover, larval dispersal was still occurring four days after the commencement of the first experiment (Section 5.3.2). It would be useful to run the larvae for longer on the servosphere to determine exactly how the larvae behave (Section 6.1).

It was hypothesised that as a larva nears pupariation the larva's speed would decrease as moulting hormones are produced initiating pupariation behaviour (Thummel, 1996; Beckstead *et al.*, 2005). While the hormone ecdysone is supressed during the larval stage there is a spike in its production prior to pupariation as this hormone is known to be involved in the initiation of the pupariation stage of metamorphosis (Thummel, 1996; Beckstead *et al.,* 2005). Therefore, it was hypothesised that this hormonal change would not be instant, but fairly steady due relatively gradual rise in the titre levels of ecdysone, present in the haemolymph, over a two day period (Shaaya and Sekeris, 1965). Consequently, the results of Section 5. 3. 1 were expected and the results of Section 5.3.2 were unexpected. Section 5.3.1 observed three single larvae, run over one hour, therefore the data examined the more minute changes in a larva's speed over a short time period, whereas the results of Section 5.3.2 examined the changes in larval speed over 4 days. One explanation for the dichotomy between the first two experiments is that the larvae tire after relatively short time periods, 5-10 minutes, and subsequently their speed decreases. Perhaps if the larvae in the second experiment had been observed over longer periods this

behaviour of fluctuating speed could have been seen. The results of 5.3.2 were even more unexpected, as half of the original population had pupariated in both populations of *C. vicina* and *P. terraenovae* (Figure 5.5.a and Figure 5.5.b), though the latter pupariated two days before the former. This means that the larvae of both of these species were moving at a constant, if not slightly increasing, rate up until pupariation. Of course, the larvae were only run once a day for 5 minutes and therefore between each larva's final run and their pupariation there could have been up to 24 hours, during which time perhaps larval speed would have decreased. Ideally each larva would have been run continuously until pupariation occurred to test this theory, however this may not be possible without more servospheres and operators.

The difference in the average speed noted between *C. vicina* and *P. terraenovae* was as predicted. At full length *Calliphora vicina* larvae are slightly larger, around 20 - 22 mm, whereas *P. terraenovae* larvae are around 18 - 20 mm (Smith, 1986). It has been suggested that dispersal speed increases as a function of larval length (Charabidze *et al.*, 2008). Therefore, it was expected that the average speed of *C. vicina* would be faster than that of *P. terraenovae*. However, it was observed that *C. vicina* ($\bar{x}$=4.08 mm/s) was almost twice as fast as *P. terraenovae* ($\bar{x}$=2.36 mm/s). Perhaps the size difference, combined with the tendency of *P. terraenovae* not to disperse far from their feeding substrate further explains this observation (Section 3.3.1). The author suggests that as *P. terraenovae* have evolved as one of the few Calliphorid species to not disperse far from their feeding substrate, this evolution may have compromised their ability to move at the speed of other Calliphorid species, such as *C. vicina*.

To the author's knowledge, the current literature concerning servosphere experiments with podous insects has not examined speed in the same way as this study has. Insect walking speed has been examined in response to stimuli and walking speed usually increases when an insect is presented with an attractive odour (Arnold *et al.*, 2016). The average walking speed of *Rhodnius prolixus* increases from 30 mm/s to 45 mm/s in the presence of human host metabolites, CO_2 and NH_3 (Otálora-Luna *et al.*, 2004), while the average walking speed of *Amblyomma variegatum* decreases from 16 mm/s to 11 mm/s in the presence of their pheromones as their walking behaviour changes to better sense the pheromones (McMahon and Guerin, 2000). No studies however assess the changes in walking speed over time in the absence of external stimuli and therefore there are no other studies with which to compare the results of this experiment.

5.4.2 Response of Calliphora vicina to light

The final result that was recorded in this Chapter is the negative phototaxis exhibited by *C. vicina* larvae. The negative phototaxis of Calliphorid larvae is well known and it is often described as a driving force in post-feeding dispersal, as the larvae search for a dark place prior for pupariation (Hinnemann *et al.*, 2010). The larvae possess light receptors, called ocelli, that enable them to detect light and thus navigate away from it (Byrd and Castner, 2009; Hinnemann *et al.*, 2010). The search for a dark place prior to pupariation may be due to the need to find a location that will be undisturbed for the duration of their pupariation. The results outlined in Section 5.3.3 show very clearly the negative phototactic behaviour of all of the larvae tested (there was one larva in Figure 5.7.c that initially turned towards the light, but by the end of the 5 minute run, the larva was moving directly away from the light source). The onset of negative phototactic behaviour appears to be almost instantaneous as all of the larval turns (90° or 180°) occurred within five seconds of the introduction of the light source and the start of the run. The behaviour was expected, but the speed and predictability of the behaviour was much greater than anticipated.

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In conclusion, some useful results were obtained using the servosphere apparatus and many more questions raised (Section 6.1.5). The author has demonstrated the ability of the servosphere to be used in this field of study and thus opened an avenue for future experiments that utilise this apparatus (Section 6.1.5).

6: Discussion

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6.1 Basis of the study

Once the study began it was decided to focus on the most important aspects that affect post-feeding larval dispersal, as due to time constraints not all aspects of dispersal could be examined. The most important aspects identified were:

- The horizontal distance dispersed by post-feeding larvae
- The vertical distance dispersed by post-feeding larvae
- The aggregation of post-feeding larvae prior to pupariation
- The effect of the dispersal substrate on post-feeding larval dispersal

These aspects were determined as the most significant as they directly affect the location (horizontal and vertical distance of dispersal) and distribution pattern of the resulting puparia and therefore have the most important practical implications. The results will help in informing the best methods for locating the dispersing post-feeding larvae and resulting puparia at deposition sites. The current literature has also highlighted further research into these aspects as being a high priority to enhance the knowledge of forensic entomology to enable more accurate estimations of minPMI (Greenberg, 1990; Godoy *et al.,* 1996; Gomes and von Zuben, 2005; Arnott and Turner, 2008; Lewis and Benbow, 2011). Once the present study began the author was given the opportunity to use a servosphere to examine insects of forensic importance for the first time. The servosphere experiments enabled the examination of post-feeding larval speed and phototactic responses with more precision than has been possible previously (Chapter 5).

It is essential to determine the horizontal distance dispersed by post-feeding larvae to be able to locate the dispersing larvae and/or puparia at deposition sites. Some studies have shown that certain species of larvae may travel up to 50 m from their origin, although the species were not specified (Byrd and Castner, 2009), while other studies have noted that some species do not move from the body at all and puparia can be found under the body and in the crevices of clothes, such as *Protophormia terraenovae* (Pohjoismäki *et al.*, 2010) and other unspecified species (Amendt *et al.*, 2007).

It is also important to determine the vertical depth dispersed by post-feeding larvae when collecting dispersing larvae and puparia at a deposition site, as the collector of the entomological

evidence needs to know how deep in the dispersal substrate to look for the evidence. Burial depths of up to 32 cm deep have been recorded (*Chrysomya megacephala* in wood shavings) (Gomes *et al.*, 2005), although the majority of sources agree that the puparia of most species of blow fly can usually be found in a natural environment within the top 5 cm of the burial substrate (Greenberg, 1990; Godoy *et al.*, 1995; Byrd and Castner, 2009).

The possible aggregation of post-feeding larvae prior to pupariation could lead to a particular distribution of puparia in the dispersal substrate. This aggregation behaviour results in a pattern of 'clumped' puparia, with an absence of puparia in other parts of the sampling area. Some species aggregate during their dispersal and different species have been shown to aggregate more than others, e.g. *Cochliomyia macellaria* which aggregates more than *Chrysomya megacephala* and *Chrysomya putoria* (Godoy *et al.*, 1996). The post-feeding larval aggregation of some blow fly species, such as *L. sericata*, has been observed to be active (Section 1.5.1.4; Boulay *et al.*, 2013). One study suggests that the ability of post-feeding larvae to detect other dispersing larvae in the dispersal substrate aids in their aggregation prior to pupariation (Holmes *et al.*, 2013).

As a highly variable physical factor, the composition of the dispersal substrate must affect the post-feeding larva's ability to disperse both vertically and horizontally. This topic, however, has again been understudied. While some studies do indeed mention the dispersal substrate as a cause of certain dispersal behaviour (such as waterlogged soil associated with longer horizontal dispersal distances) (Lewis and Benbow, 2011), most studies overlook this factor and one even stated that the saturation of the substrate with water has no effect on the dispersing larvae (Miller, 1929). This study aimed to examine all of the aspects, outlined above, separately and simultaneously to determine the extent of the effects of species and dispersal substrate on the horizontal and vertical dispersal behaviour and aggregation prior to pupariation.

The species chosen for this study were *Calliphora vicina, C. vomitoria, L. sericata* and *P. terraenovae*. These species were chosen as they are the most common species of forensically important Calliphoridae in the UK (Smith, 1986; Byrd and Castner, 2009). *Calliphora vicina* is frequently the most abundant of all the species present at crime scenes (Smith, 1986; Szpila *et al.*, 2014; Hall *et al.*, 2015). *Calliphora vomitoria* (Smith, 1986; Szpila *et al.*, 2014)and *L. sericata* (Smith, 1986; Szpila *et al.*, 2013) are also very common at UK crime scenes. *Protophormia terraenovae* (Smith, 1986) is found throughout the UK and is present in around 8 % of cases in the UK (Hall and Mactaggart, 2018); this species was also chosen because it is more often described as a species that does not disperse from the body at a crime scene, but pupariates on

or very close to the body itself (Erzinçlioğlu, 1996; Gennard, 2007). *Protophormia terraenovae* therefore, was determined as a good species in the study of post-feeding larval dispersal to compare with the other species, which are generally acknowledged to disperse away from the food source to pupariate.

The experiments using the servosphere apparatus offered a unique opportunity to examine a single dispersing larva at a time, under highly controlled conditions (Fig 5.1.a and 5.1.b). The apparatus enabled the study of the post-feeding larval speed and distance dispersed over time of *C. vicina* and *P. terraenovae*. More detailed examination of calliphorid post-feeding larval dispersal speeds is useful in the field of forensic entomology, as estimating the time that has elapsed once the larvae have left the body enables more accurate estimations of minPMI to be determined (Charabidze *et al.*, 2008). Using the servosphere, it was also possible to examine the phototactic response of post-feeding larvae in a precise manner as the larval tracks produced very clearly show the larval response. Again, expanding the knowledge of calliphorid post-feeding larval behaviour is useful for the field of forensic entomology.

6.2 Summary of results

The laboratory experiments (Chapter 3) were conducted to examine the factors of post-feeding larval dispersal separately (i.e. horizontal dispersal, vertical dispersal, the effect of dispersal substrate and the behaviour of different species), such that each factor was examined as an 'extreme' disparate situation that would not normally occur independently in a natural setting.

The 6 m gutter experiments outlined in Section 3.2.2 allowed the post-feeding larvae to disperse horizontally and in one direction only (although the larvae could disperse the length of the gutter, turn 180° and disperse back towards the origin). Therefore, the results outlined in Chapter 3 can be seen as the 'extreme' version of what is possible and can be used as a guide to extend the limits of crime scene collection protocols. During the 6 m gutter experiments (Section 3.3.1) the post-feeding larvae were able to disperse unimpeded throughout the full length of the gutter and yet in all cases that used a dispersal substrate (CVIC WLG A-C, CVIC SAW A-C, PTER SAW A-C) the majority of puparia (> 50 %) were recovered within the first 3 m of the gutter (apart from CVIC SAW A, where >50 % of puparia were recovered within the first 4 m). The substrates used in these experiments were 'ideal' pupariation environments: sawdust and soil. Sawdust was chosen as a dispersal substrate as it is commonly used in controlled experiments (Greenberg and Szyska, 1984; Gomes *et al.*, 2006; Mai and Amendt, 2012) and soil was chosen to be representative of

an outdoor deposition site. It was originally thought that sawdust would provide an 'easier' medium through which to disperse and therefore differences would be observed between sawdust and soil. However, this was not the case (Section 3.3.1), although there were differences recorded in the distance dispersed with the bare gutter in comparison to the other dispersal substrates (soil and sawdust). The larvae tended to disperse the full length of the 6 m gutter, where over half of the puparia in the bare plastic runs were recovered from the first and last 50 cm of the gutter (Section 3.3.1), a significant difference when compared with the other substrates. Previous studies, such as Byrd and Castner (2009), have demonstrated that dispersing larvae are able to move up to 50 m from the food source, although the reason for extended dispersal and the species was not suggested. If a substrate is not suitable for larval penetration and subsequent pupariation, post-feeding larvae have been shown to continue dispersal until an adequate site is found, for example moving up to 26 m on waterlogged soil until suitable soil was found (Lewis and Benbow, 2011). Moreover, Robinson *et al.* (2018) showed that pupariation mediums unsuitable for larval penetration, such as plastic flooring, resulted in further post-feeding dispersal distances in comparison to suitable pupariation mediums, such as sawdust or carpet, which resulted in much shorter dispersal distances (experiments conducted using *L. sericata*). Given the results of the bare gutter experimental runs and the current literature surrounding this topic, the dispersing larvae would most likely have dispersed the full length of a gutter much longer than 6 m as the bare gutter was not an ideal pupariation medium. Given a more suitable pupariation environment (two types of soil or sawdust) the dispersing larvae exhibit a preference for limited dispersal (Section 3.3.1). This behaviour makes evolutionary sense, as limiting their energy expenditure when a suitable pupariation medium is available should enable shorter pupariation periods and the emergence of larger adults (Arnott and Turner, 2008; Mai and Amendt, 2012). It is important to note that the composition of the dispersal substrates used during this study (soil and sawdust) were homogenous and friable, thus allowing unimpeded larval movement through the substrate whereas in a natural setting the dispersal substrate would rarely be homogenous and therefore the speed and distance of the dispersing larvae will be highly variable (Cammack *et al.*, 2010). According to the results of this study, in real-life situations where the substrate is homogenous, friable and not impenetrable, larvae will most likely disperse up to 4 m from the food source and where the dispersal substrate is impenetrable larval dispersal may occur on the dispersal substrate at least 6 m (Section 3.3.1) and up to 26 m (Lewis and Benbow, 2011) or 50 m (Byrd and Castner, 2009) from the food source. Therefore, when searching for post-feeding larvae, puparia or empty puparia cases at a deposition site, the type of dispersal substrate should be considered to help determine how far from the body to search for the entomological evidence (Section 6.1.4).

During all of the experimental runs of the vertical dispersal experiment over half of the puparia were recovered within the top 9-27 cm (Section 3.3.3). However, the maximum depth reported from each experiment was at least 36 cm, and during one experiment, was 66 cm deep (Figure 3.28.c). These experiments show that dispersing post-feeding larvae, when in non ideal conditions, are more than capable of burrowing much deeper than the 5 cm generally reported in the literature (Greenberg, 1990; Godoy *et al.*, 1995; Byrd and Castner, 2009). Balme *et al.* (2012) showed that when *Cochliomyia macellaria* and *P. terraenovae* puparia were manually buried, they all successfully emerged after burial up to 50 cm deep, moreover 40 % of *C. macellaria* successfully emerged after being buried 120 cm deep. The adult emergence stage was not examined in the present study, but Balme *et al.*, (2012) demonstrated that the specimens used during the vertical dispersal experiment (Section 3.3.3) would most likely have emerged successfully (assuming that the species examined in the present study behave similarly to *C. macellaria*). Post-feeding larvae, when searching for a suitable pupariation site, may burrow deeper in the soil when only vertical dispersal may be possible (i.e. a crack in the pavement that exposes soil). Other advantages of increased vertical dispersal included the evasion of parasites. In this regard Frederickx *et al.* (2014) demonstrated that puparia situated deeper in the substrate (4 cm was deepest depth tested by Frederickx *et al.* [2014]) are less susceptible to parasitism, by parasitoids like *Nasonia vitripennis.* During the simultaneous horizontal and vertical experiment (Section 3.3.4) the dispersing larvae were able to move horizontally and vertically unimpeded. Due to the construction of the apparatus (Figures 3.6.a, 3.6.b and 3.6.c) the dispersing larvae were still limited to horizontal and vertical dispersal in one plane only. In all of the experimental runs (Section 3.3.4; CVIC COM A-C and CVIC SAW A -C) puparia were recovered throughout the horizontal plane and 50-80 % of the puparia were recovered from the top 5 cm of the dispersal substrate. During the field experiment (Chapter 4) the majority of puparia were recovered from within the top 5 cm of the substrate during each run: 82 % (Figure 4.3.a and 4.3.b), 89 % (Figure 4.4.1 and 4.4.b) and 99 % (Figure 4.5.a and 4.5.b) respectively. These experiments highlight that when the dispersing larvae are given a choice of horizontal and vertical dispersal, horizontal dispersal is favoured and the majority of puparia are recovered from the top 5 cm of the dispersal substrate- a finding also supported in the literature (Godoy *et al.*, 1995; Cammack *et al.*, 2010). Therefore, although post-feeding larvae are capable of great vertical dispersal, where horizontal dispersal is possible, the vast majority of larvae, puparia and empty puparial cases should be recovered from within the top 5 cm of the soil (Section 3.3.4, Section 4.3.1). However, if circumstances at a deposition site limit horizontal dispersal then any possible vertical dispersal substrate should be examined up to 27 cm deep (> 50 % larvae recovered within the top 27 cm for all vertical experiments [Section 3.3.3]). Therefore, when searching for post-feeding larvae, puparia or empty puparia cases at a deposition site, the dispersal substrate should be used to

help determine how deep in the substrate to search for the entomological evidence (Section 6.1.4).

The results of the experiments outlined in Sections 3.3.1 and 3.3.2 suggest that aggregation prior to pupariation occurs. Puparia that were recovered from the same sampled section of the horizontal dispersal experiments (Sections 3.3.1 and 3.3.2) were often found directly touching each other. This suggests a preference of post-feeding larvae to either actively seek out puparia or to aggregate with other post-feeding larvae that they passively come into contact with prior to pupariation. The results of this study support those of Fouche *et al.* (2018) that showed that both *C. vomitoria* and *L. sericata* post-feeding larvae exhibited a preference for actively aggregating in areas previously marked by other larvae of both species. Moreover, the results of the experiments conducted in the 6 m gutter with no substrate showed that the larvae dispersed the full 6 m of the gutter in search of a suitable pupariation site, and pupariated mainly at either end. This is possibly due to the thigmotactic response of post-feeding larvae (Holmes *et al.*, 2013), whereby the larvae concentrate at the ends of the gutter, where chance of contact is greatest. Not only is the chance of contact with the apparatus itself greater at either end of the gutter, but by forming aggregates the larvae increase their chance of touching each other. This is even more clear when examined in conjunction with the results from the bare plastic experiments in Section 3.3.1 and Section 3.3.2, where strong puparial aggregates were formed in every experimental run conducted with no dispersal substrate. Holmes *et al.* (2013) demonstrated that pre-puparial larvae in the presence of no burial substrate will aggregate together and suggested that this behaviour is a thigmotactic response to the surrounding puparia and pre-puparial larvae. Lima *et al.* (2009) suggest that neighbourhood interactions between larvae predispose them to aggregate prior to pupariation as the post-feeding larvae are able to detect other larvae and puparia in the dispersal substrate.

The aggregation of individuals of a population is well documented throughout the animal kingdom, e.g. in bacteria, arthropods, fish, birds and mammals (Veit and Hunt, 1991; Parrish and Edelstein-Keshet, 1999; Krause *et al.*, 2000; Das *et al.*, 2010). The main reported benefits of such aggregations include an increase in reproductive success, conservation of energy, an increase in food finding ability and protection from predators (Parrish and Edelstein-Keshet, 1999). The most likely potential benefit for the aggregation of blow fly larvae prior to pupariation is protection from predators and parasitoids (Reigada and Godoy, 2005; Lewis and Benbow, 2011). During all stages of their development blow flies can be predated on by many different types of predators, including insectivorous birds, mammals, beetles, arachnids and myriapods (Nuorteva, 1970; Putman, 1983; Kočárek, 2001; Merfield *et al.*, 2004; Roberts, 2008). Blow flies can also be

parastised during any stage of their development by organisms such as: Hymenoptera and Diptera (Andrade *et al.*, 2002; Amendt *et al.*, 2004; Cammack *et al.*, 2010; Frederickx *et al.*, 2014). One mechanism of protection from predators that is most applicable to blow fly puparia is the idea that an increase in prey numbers decreases the likelihood of any one individual being attacked, thus aggregations convey protection to individuals of the population (Turchin and Kareiva, 1989). This theory is supported by Cammack *et al.* (2010), who proposed that the increased aggregation of *L. sericata* in the presence of parasitoids conveyed a 'safety in numbers' strategy to the blow fly. The study by Reigada and Godoy (2005) also supports this theory as *Chrysomya megacephala* post- feeding larvae were shown to only aggregate in the presence of predatory species of blow fly larvae.

With regards to locating larvae at a deposition site, the results of this study highlight the importance of the dispersal substrate. The 20 x 20 cm sampling technique employed during the field experiment (Chapter 4) demonstrated that at an outdoor scene with a soil based dispersal substrate, puparial aggregations can be seen and therefore all relevant entomological evidence was collected with this sampling technique. However, the results of the experiments conducted in the 6 m bare gutter (Section 3.3.1) suggest that larval aggregations can be found at least 580 cm from each other (puparial aggregations were recovered from either end of the gutter). Therefore, when searching for post-feeding larvae, puparia or empty puparia cases at a deposition site, the type of the dispersal substrate (e.g. smooth plastic or loose soil) should be used to help determine how far from the body to search for the entomological evidence, as larval, and the subsequent puparial, aggregations could result in large areas with no entomological evidence (Section 6.1.4).

The literature suggests that the distance larvae may disperse from their food substrate is species specific and this is most likely due to the different pupariation environments required by different species (Godoy *et al.*, 1995). The difference in behaviour exhibited between species was examined in this study. It was observed that the only difference between the species examined was the horizontal dispersal behaviour of *P. terraenovae.* During two of the experimental runs using *P. terraenovae* post-feeding larvae (Section 3.3.1., PTER SAW B and C), 100 % of the puparia were recovered from the first 2 m of the 6 m gutter. The results for the other species in the 6 m run showed that all of the puparia (100 %) were recovered from between 4 and 6 m of the origin. These results support those reported in the literature, where *P. terraenovae* does not disperse far from the body and in fact the puparia are often found on the body itself (Erzinçlioğlu, 1996; Pohjoismäki *et al.*, 2010). Nevertheless, the results of one of the experimental runs (Section 3.3.1., PTER SAW A) showed that puparia of *P. terraenovae* were recovered up to 5.5 m from the

origin and the overall distribution pattern of this run was much more similar to those of the other species. This is a very interesting result as it demonstrates the capability of *P. terraenovae* to disperse as far horizontally as the other main UK species under certain circumstances. There were no known biotic or abiotic differences during this run (PTER SAW A), compared with the other two (PTER SAW B and C) and therefore no explanation can be offered for the difference in results. Thus, without further experimentation, all that can be concluded is that *P. terraenovae* is capable of dispersing much further horizontally than previously assumed. The recorded difference in the results was either due to an external factor unknown to the author or a product of the biology of the organism itself. In the literature *P. terraenovae* is known not to disperse far from their feeding substrate and is often recovered as puparia on the feeding substrate itself (Erzinçlioğlu, 1996; Gennard, 2007; Pohjoismäki *et al.*, 2010). Greenberg, (1990) showed that during the same experiment *C. macellaria* individuals from the same population that were exposed to the same conditions pupated at different distances from the origin. Roughly half pupariated on the source, while half travelled up to 2.4 m from the source to pupariate. Greenberg's (1990) study may help to explain the results recorded for *P. terraenovae* in the present study as perhaps the differences in the results of both studies are the product of the biology of the organisms themselves. Therefore, when searching for post-feeding larvae, puparia or empty puparia cases at a deposition site, the species of blow fly present should be used to help determine how far from the body to search for the entomological evidence, and moreover when a species that is known not to disperse from the body is present (such as *P. terraenovae*) the same practices should be employed as with the other species, in case the post-feeding larvae have dispersed further than usual (Section 6.1.4).

As the laboratory experiments represented extreme scenarios, the expected results from the field experiments were that horizontal and vertical larval dispersal would occur, but the overall distances and depths dispersed would be less than recorded during the laboratory experiments. This was indeed what was observed in the field, therefore, the results of the field experiments corroborate the laboratory findings. Three field experiments were conducted to assess the above-mentioned factors in a more 'real-life' setting. The laboratory studies examined all factors separately or two at a time (Section 3.2.5 and Section 3.3.4), but the field experiments offered an environment to observe all factors simultaneously (horizontal and vertical dispersal and 360° about the point of origin) and assess their effect on the behaviour of *C. vicina* post-feeding larvae. The field experiment results most closely reflected the simultaneous horizontal and vertical experiment (Section 3.3.4), where the post-feeding larvae were given the choice to move vertically or horizontally. In each experimental run, over 80 % of the puparia were recovered from the top 5 cm of soil, while horizontal dispersal over 40 cm from their origin did not occur.

The final set of experiments were carried out using a servosphere (Chapter 5), which allowed the study of one post-feeding larva at a time in a highly controlled setting (Figures 5.1.a and 5.1.b). This level of control was not possible during the other laboratory experiments (Chapter 3). The servosphere was used to examine the speed, distance per unit time and phototaxis of *C. vicina* and *P. terraenovae*. The experiments conducted using this set up were especially novel as this study represented the first use of a servosphere to study apodous insects. Moreover, in the field of forensic entomology it is often not possible to study the insects in a highly controlled setting, and the servosphere offered this opportunity.

One of the factors examined with the servosphere was the speed at which post-feeding *C. vicina* and *P. terraenovae* larvae disperse. Section 5.3.1 observed three single larvae, run over one hour, therefore the data examined the more minute changes in a larva's speed over a short time period, whereas the results of Section 5.3.2 examined the changes in larval speed over 4 days. During the second experiment, a mean speed of 4.08 mm/s (14 m/hr) was recorded for *C. vicina* and a mean speed of 2.36 mm/s (8.50 m/hr) for *P. terraenovae* (Section 5.3.2). The dispersal speeds recorded represent the dispersing ability of *C. vicina* and *P. terraenovae* on smooth surfaces. The significantly higher speeds of *C. vicina* versus *P. terraenovae* were anticipated as the fully grown larvae of the former (20-22 mm) are generally larger than those of the latter (18-20 mm) and it has been suggested in the literature that dispersal speed increases as a function of larval length (Charabidze *et al.*, 2008). During the second experiment (Section 4.3.2), *C. vicina* dispersed an average distance of 4927 mm (4.92 metres in 20 minutes [4 x 5 minutes]) and *P. terraenovae* dispersed an average distance of 2920 mm (2.92 metres in 20 minutes [4 x 5 minutes]). Again, the further overall average distance dispersed by *C. vicina* larvae was anticipated, due to the larger size of their larvae compared with *P. terraenovae*. These results reiterate the considerable dispersing capability of calliphorid post-feeding larvae on a smooth surface. It is important to note that there was a high level of intraspecific variation in the speeds and track lengths recorded during this experiment (Section 5.3.2). Whether this variance is representative of the two species as a whole or due to influencing factors that the author was unaware of, is currently unknown. Further research in this area should help to clarify the reason behind the variance (Section 6.1.5).

The author hypothesised that as the post-feeding larvae of *C. vicina* and *P. terraenovae* neared pupariation the larval speed would decrease as moulting hormones are produced initiating pupariation behaviour (Thummel, 1996; Beckstead *et al.*, 2005). There is an increase in the production of the hormone ecdysone prior to pupariation and it is supressed during the larval

stages and this hormone is known to be involved in the initiation of the pupariation stage of metamorphosis (Thummel, 1996; Beckstead *et al.*, 2005). Therefore, it was hypothesised that this hormonal change would not be instant, but relatively gradual due to the rise in the titre levels of ecdysone, present in the haemolymph, over a two day period (Shaaya and Sekeris, 1965). Consequently, the results of Section 5. 3. 1 were expected and the results of Section 5.3.2 were unexpected. The author suggests that the contrast between the first two experiments may be because the larvae tire after relatively short time periods, 5-10 minutes, and subsequently their speed decreases. Perhaps if the larvae in the second experiment had been observed over longer periods this behaviour of fluctuating speed could have been seen. The results of the second experiment show that the larval speed of both species does not decrease as time increases, in fact the larval speed is slightly increasing as can be seen by the linear regression lines fit to the graphs (Figures 5.5.a and 5.5.b). This must mean that there is not a gradual increase in the production of the hormone ecdysone prior to pupariation, but that the hormonal change must be more rapid, unless a threshold of particular hormones, such as ecdysone, is required to initiate pupariation. The reason for the increase in speed over time is unknown, but the author hypothesises that as post-feeding larvae near pupariation without having encountered an appropriate pupariation site, more energy is expended to increase their speed and thus increase their chance of locating a suitable location. Therefore, when searching for post-feeding larvae, puparia or empty puparia cases at a deposition site, if the dispersal substrate is smooth (such as the smooth plastic of the servosphere), speeds of up to 14 m/hr for *C. vicina* and 8.50 m/hr for *P. terraenovae* should help determine how far from the body to search for the entomological evidence (Section 6.1.4).

The final factor that was assessed during the servosphere experiments was the phototactic response of post-feeding *C. vicina* larvae. This is a phenomenon that is well known and widely stated in the literature as it can be observed during laboratory studies and in the field (Hinnemann *et al.*, 2010). However, to the author's knowledge, this behavior has not previously been studied in such a precise and controlled way. The results of the experiment were as expected, where a strong negative phototactic response was exhibited by all larvae, within 5 seconds of the introduction of the light source (Section 5.3.3).

6.3 Strengths and limitations of the study

Initially this study aimed to examine nine factors that affect and are affected by post-feeding blow fly larval dispersal (Section 6.1.1), however due to time constraints the study focused on only four factors of dispersal (Section 6.1.1). This study was, therefore, not so much a complete study of

calliphorid post-feeding larval dispersal, rather an initial examination of this complex stage of their development as it was not possible to the variety and quantity of experiments that would have been ideal.

One of the main limitations of this study were the lack of repeated runs in some experiments. For example, more repeats of the 6 m gutter experiment using *P. terraenovae* would have been beneficial as some unexpected results were obtained (Section 3.3.1). The study could have been expanded to examine more dispersal substrates commonly encountered at scenes, for example, different types of soil encountered at outdoor cases and more indoor floorings, such as different carpets and rugs.

Some of the most interesting results of this study were obtained using the servosphere and there is huge scope to expand this area of research. Again, more experimental runs using the servosphere would have been beneficial. The servosphere could also have been used to examine some additional factors of post-feeding larval dispersal, such as an 'innate directional preference' exhibited by the dispersing larvae (Section 1.5.3).

The study could also have benefited from running all of the experiments using more UK blow fly species, such as *L. sericata* and *C. vomitoria*, but due to time constraints only a few runs were conducted using these species.

6.4 Implications for the field of forensic entomology

The main implication of this study is the need to examine and develop a search strategy at each site according to the unique local circumstances. The post-feeding larval dispersal stage of Calliphoridae is highly variable and not necessarily wholly predictable. For example, the differences in the results of the 6 m horizontal dispersal runs of *P. terraenovae*, where 100 % of the puparia in two of the runs (PTER B and C) were recovered from within the first 2 m, whereas puparia were recovered up to 5.5 m from the origin in the other run (PTER A [Section 3.3.1]). If post-feeding larvae and/or puparia are not recovered from a deposition site the resulting minPMI estimation, calculated using the larvae collected from the body, will most likely be an underestimation of the true PMI. It is therefore essential to ensure that these older stage calliphorids are recovered from a crime scene, which may not occur if some current recommended crime scene protocols are not followed (Lewis and Benbow, 2011).

Currently the recommendation for collecting entomological evidence from a deposition site is to search 360 ° of the area surrounding the body within a 10 m radius (Amendt *et al.,* 2007; Byrd and Castner, 2009). If these protocols are followed, post-feeding larvae and puparia may be overlooked. Amendt *et al.* (2007) also advises that at outdoor scenes, soil samples should be taken up to at least 2 m from the body and to a depth of 10 cm or more, depending on the circumstances, and at an indoor scene it is advised to check different rooms, not just the room in which the body was discovered, for dispersed/dispersing larvae and/or puparia/empty puparial cases. This advice is not easy to follow without forensic entomological expertise. The value of entomological evidence collected at a deposition site is further complicated by the fact that often the evidence is not collected by a forensic entomologist due to the current practice of forensic streamlining used in the UK (Hall *et al.,* 2015). In the UK, more often SOCOs or CSIs are employed to collect all of the forensic evidence. Ideally a forensic entomologist should be present at all scenes to collect all of the relevant entomological evidence, using current crime scene protocols, combined with their expertise (i.e. knowledge of the biology, ecology and behaviour of forensically important insects) and the advice given in the current literature, including this study. Therefore, an easy to follow instruction manual should be available and disseminated to all UK SOCOs and CSIs, that does not require extensive entomological training and knowledge to interpret (Lewis and Benbow, 2011; Magni *et al.,* 2013). Below is some suggested information that should be included, concerning the collection of post-feeding larvae and puparia at deposition sites.

Firstly, it is important to reiterate that the collection of entomological evidence from a deposition site should be treated in a situation specific manner, i.e. there is not a 'one size fits all' collection protocol. Also, in all indoor and outdoor cases the wider area should be searched for possible alternative sources of post-feeding larvae and puparia, such as other decomposing mammals.

Locating dispersing larvae and puparia at indoor deposition sites:

- Where the dispersal substrate is smooth, preventing larval penetration (like the plastic gutters), the area should be checked 360 ° around the body and at least 6 m from the body itself (Section 3.3.1) and where there are no obstacles present that would interfere with dispersal (for example in very large rooms, such as a warehouse), as much of the dispersal substrate as possible should be examined, as some species have been shown to travel at speeds of up to (19.84 m/hr) (Chapter 5).
- Where obstacles are present, such as the edge of a room or furniture, these areas should be examined more thoroughly due to the thigmotactic response of dispersing larvae

(puparia were recovered from either end of the bare gutters, presumably due to positive thigmotaxis; Sections 3.3.1 and 3.4) (Holmes *et al.*, 2013).

- Where the dispersal surface is smooth, puparia are likely to be recovered in aggregations at the edge of obstacles (more so than the usual aggregation behaviour observed in porous dispersal substrates, such as soil). Therefore, there may be many areas within an open area with no puparia due to extreme aggregation behaviour of puparia under these circumstances (Chapter 3; Section 3.4).

Locating dispersing larvae and puparia at outdoor deposition sites:

- The area should be checked 360 ° around the body and at least 4 m from the body itself (less horizontal distance compared to indoor scenes due to the penetrability of most outdoor dispersal substrates [Section 3.3.1]) and sample blocks of 20 x 20 cm^2 should be excavated (Section 4.2.1).
- Where horizontal dispersal occurs unimpeded, depths of 5 cm should be sufficient for the recovery of over 80 % of the puparia (Section 3.3.3).
- Where horizontal dispersal might be impeded, depths of up to 20 cm should be excavated and this should be sufficient for the recovery of > 75 % of puparia (Section 3.3.3 [experiments conducted using soil, as sawdust is not comparable to most natural settings]).

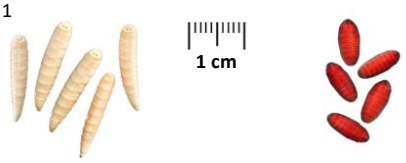
The information on page 17 is prepared as an addition to the current guides on collecting and preserving entomological evidence (Amendt *et al.,* 2007; Byrd and Castner, 2009; Hall *et al.,* 2012), and could be included for example under the 'Where to collect entomological evidence' in Amendt *et al.*'s (2007) guide.

6.5 Protocol for crime scene investigators:

When searching for dispersing/dispersed larvae and puparia/empty puparial cases:

Larvae (or maggots) will usually leave the body to find somewhere suitable to pupariate, how far the larvae travel and how deep they burrow into the substrate depends on a few factors that are discussed below. Please note that the 'dispersal substrate' refers to the material that the larvae move on, e.g. carpets, tiles, soil etc. For an example of what larvae and puparia look like please see Figure 1 (puparia and empty puparial cases look very similar)

Figure 1



Larvae (maggots) **Puparia**

Locating dispersing larvae and puparia at indoor deposition sites:

Is the dispersal substrate smooth (e.g. smooth plastic floor)?

- If no, then the area should be checked for larvae and/or puparia **360 °** around the body and up to **4 m** from the body itself.
- If yes, then the area should be checked for larvae and/or puparia **360 °** around the body and at least **6 m** from the body itself, even further in a very large room (e.g. a warehouse), up to the peripheral walls.

Please note:

- Where **obstacles** are present, such as the edge of a room or furniture, these areas should be examined more thoroughly as larvae tend to congregate in these areas.
- Where the dispersal surface is smooth, puparia are likely to be recovered in '**clumps**', i.e. many larvae/ puparia in one small section (i.e. 10 cm^2). Therefore, there may be many areas within an open space with no puparia due to the 'clumping' behaviour or larvae. Thus, a thorough examination of the site is required to ensure such 'clumps' are not overlooked.

Locating dispersing larvae and puparia at outdoor deposition sites:

In general:

- The area should be checked **360 °** around the body and at least **4 m** from the body itself and sample sections of **20 x 20 cm^2** should be excavated, up to **5 cm** should be sufficient for the recovery of over 80 % of the puparia.
- If obstacles are present (e.g. tree trunks) depths of up to **20 cm** should be excavated as this should be sufficient for the recovery of over 75 % of puparia.

6.6 Future avenues of research

As previously stated, this study could be improved with more repeats of some of the experiments, especially concentrating on other main UK calliphorid species of forensic importance (the majority of the study has focused on *C. vicina* and *P. terraenovae*). Therefore, future studies that focused on these species would be complementary to the work set out in this study. Specifically, more runs of the vertical dispersal experiment (Section 3.3.3) and the simultaneous horizontal and vertical experiment (Section 3.3.4) should be conducted using *C. vomitoria, P. terraenovae* and *L. sericata* as the experiments in this study only examined *C. vicina*. Also, more experimental runs in the 6 m horizontal dispersal apparatus (Section 3.3.1) using *C. vomitoria* and *L. sericata* are required, as these species were not utilised in this study. Experiments that examine horizontal dispersal in gutters that are longer than 6 m should be conducted, to determine the maximum horizontal dispersal possible by each main species in different dispersal substrates and, particularly, using no substrate.

The experiments conducted using the servosphere were limited, due to time constraints. The servosphere offers a unique opportunity to examine calliphorid larva individually. The apparatus also enables the investigator to introduce different stimuli and record the larval responses. For example, one experiment that would be of considerable forensic interest would be to examine the post-feeding larval response to the volatiles of a decomposing substrate, such as pork liver, and then to compare the responses of 1st, 2nd and feeding 3rd instar calliphorid larvae. The author hypothesises that the feeding larvae would be actively attracted to the volatiles, while the post-feeding larvae would be actively repelled. This hypothesis is proposed because when feeding larvae are removed from their food source and an additional food source placed close to them, they are able to detect the food and move towards it to continue feeding (personal observations). Moreover, Bhadra *et al.* (2014) showed that 1st instar larvae will move towards the source of the decomposing tissue inside a suitcase when laid as eggs on the outside of a suitcase, even passing through a closed zipper. Post-feeding larvae of most blow fly species leave their food substrate to disperse in a 360 ° pattern around the body, therefore there must be a response that directs the larvae away from the feeding substrate. Testing the chemotactic response of larvae to larval trails would also be interesting to examine. The experiments outlined in Chapter 3 have shown that aggregation prior to pupariation occurs. The literature has suggested the ability of larvae to recognise ground marking signals of other larvae of their species (Boulay *et al.*, 2013, 2015, 2016) and therefore such traces could provide an explanation for the ability of post-feeding larvae to aggregate prior to pupariation. Perhaps larvae would have a positive chemotactic response to the trails left by previous larvae and, if this were the case, then that would provide an explanation for

the mechanism of aggregation prior to pupariation. Experiments conducted to investigate the direction of post-feeding larval dispersal would also be of benefit to inform forensic entomology crime scene protocols.

There are many exciting experiments that can be conducted using the servosphere and it is the author's intention to continue work in this area. The author also intends to expand the current work that has been reported in this study, for example to examine the intraspecific variations recorded in larval speed and track length and to determine whether there are any factors, previously overlooked, that affect the larvae.

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An Investigation of Post-Feeding Larval Dispersal in UK Blow Flies

Molly Mactaggart[1,2], Amoret Whitaker[1,2], Martin Hall[2] and Keith Wilkinson[1]

Annual Meeting of the EAFE, Treviso, Italy (2017)

In order to determine the minimum post-mortem interval (minPMI) in forensic investigations, it is essential to collect the oldest insect specimens associated with the body. Blow flies are the primary colonisers of a cadaver and are therefore acknowledged as the most important insects in determining minPMI. The oldest blow fly specimens are often not found on the body itself, but have dispersed to find a suitable pupariation site, and therefore may be some distance from the body on which they were feeding. To locate these dispersing larvae and puparia, it is first necessary to know where to look for them. A literature review revealed that much information is either contradictory or missing concerning the behaviour of post-feeding blow fly larvae. This has highlighted a need for a more complete study exploring the many factors that may affect larval dispersal. Perhaps most important is to determine how far the post-feeding larvae will disperse and how deep they will burrow; in order to maximise crime scene collection efforts.

Experiments were conducted to examine the dispersal behaviour of UK blow flies, specifically the distance dispersed (horizontal dispersal) and the depth dispersed (vertical dispersal) prior to pupariation. Post-feeding *Calliphora vicina* larvae were introduced into different experimental apparatuses to explore the different factors: a 600 x 10 x 10 cm plastic channel was used to investigate horizontal dispersal; a 60 x 8 cm plastic pipe was used to look at vertical dispersal; and a 100 x 50 x 5 cm wooden apparatus was used to look at horizontal and vertical dispersal in conjunction with each other. The results suggest that in an experimental laboratory setting *C. vicina* can disperse up to 500 cm horizontally and up to 50 cm vertically. Further experiments will also be discussed.

Investigation of Post-Feeding Larval Dispersal in the UK Blow Fly: *Calliphora vicina* (Diptera: Calliphoridae)

Molly Mactaggart[1,2], Amoret Whitaker[1,2], Martin Hall[1], Keith Wilkinson[2]

Annual Student Conference, Natural History Museum, London, UK (2018)

In order to determine minPMI (minimum post mortem interval) from insect evidence it is necessary to examine the oldest specimens associated with the body and often these are not found on the body itself. Larval dispersal occurs after the feeding stage of the final, third instar. A greater understanding of this understudied stage is necessary to predict where dispersing larvae and the developing puparia are most likely to be found. Experiments were conducted looking at three factors of dispersal: the distance dispersed (horizontal dispersal), the depth dispersed (vertical dispersal) and whether the dispersing larvae aggregate prior to pupariation. Post-feeding *Calliphora vicina* larvae were introduced into different arenas to explore the different factors: a 600x10x10cm plastic arena was used to investigate horizontal dispersal and aggregation; a 60x8cm plastic pipe was used to look at vertical dispersal and aggregation; and a 100x10x10cm plastic arena was used to look at aggregation alone. The results suggest that over half of dispersing larvae do not burrow more than 10cm vertically, over half of dispersing larvae do not move more than 2m horizontally and that aggregation is occurring, producing a non-random, clumped abundance of puparia in the substrate.

The First Investigation of Blow Fly (Diptera: Calliphoridae) Post-Feeding Larval Dispersal using a Servosphere

Molly Mactaggart[1,2], Amoret Whitaker[1,2], Keith Wilkinson[2], Martin Hall[1]

Annual Meeting of the EAFE, Munich, Germany (2018)

In order to determine the minimum post mortem interval (minPMI) in forensic investigations it is essential to collect the oldest insect specimens associated with the body. Calliphoridae are typically the primary colonisers of a body and as such are considered the most important insects when determining minPMI. As post-feeding calliphorid larvae usually disperse to find a suitable pupariation site, often these specimens are not located on the body itself, but may be located some distance away. The post-feeding dispersal stage of calliphorid larvae is underrepresented in the literature and thus experiments were conducted to investigate this stage further, in order to provide information to increase the ability of crime scene investigators to locate the oldest specimens at a crime scene.

A Syntech Tracksphere LC-300 (servosphere) was used during this study to examine calliphorid post-feeding larval dispersal. The apparatus consisted of a servosphere, a CMOS camera and a control unit. The individual larvae were placed at the apex of the sphere, the camera visually tracked the insect and the servosphere then rotated, via servomotors, such that the larva's position was maintained at the apex of the sphere. In this way the servosphere allowed unimpeded larval movement in any direction. The larval tracks were recorded using TrackSphere 3.1 software, which provided both raw and partially processed data.

The main factors examined were: larval speed, total distance dispersed in discrete time periods and larval phototaxis. Prior to this study the use of servospheres had been limited to the study of podous invertebrates: this study represents the first use of a servosphere to examine apodous invertebrates. Surprisingly, the results demonstrated that over a five-day period post-feeding, the mean speed and therefore overall track length per unit time of larvae increased. As expected, the larvae showed a strong negative phototactic response.

METHODS FOR THE INVESTIGATION OF POST-FEEDING LARVAL DISPERSAL IN UK BLOW FLIES: PRELIMINARY RESULTS

Molly M. Mactaggart[1,2], Amoret P. Whitaker[1,2], Martin J. R. Hall[2] and Keith N. Wilkinson[1]

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Introduction

In order to determine the minimum time since death in forensic investigations, it is essential to collect the oldest insect specimens associated with the body (Amendt et al. 2011). Often these are not found on the body itself, but have left to find a suitable pupariation site and, therefore, may be some distance from the body on which they were feeding (Lewis & Benbow 2011). A literature review revealed that much information is either contradictory or missing concerning the post-feeding larval dispersal behaviour of blow flies. This has highlighted the need for a more complete study to consider the many factors that may affect larval dispersal. Therefore, based on published experimental studies, multiple methods were explored to determine the most appropriate to study these factors. Although the results of this study are preliminary, some patterns have been observed, including the possibility of the aggregation of puparia.

Material and Methods

Three methods were tested and a total of eight experiments conducted.

Method A: Adapted from multiple studies looking at the post feeding larval dispersal of blow flies (Greenberg 1990; Andrade et al. 2002). An acrylic channel was filled with soil, maximum 5cm deep. The soil used was taken from the Wildlife Garden at the Natural History Museum, London. Post-feeding *Calliphora vicina* larvae were introduced and left to pupariate. After one week the number of puparia recovered from each 10cm segment was recorded.

Experiments using Method A:

A1 and A2. The channel was 4m long, 6cm wide and 12cm deep. 100 larvae were introduced at the midpoint of the channel.

A3. The channel was 6m long, 6cm wide and 12cm deep. 320 larvae were introduced at the midpoint of the channel.

A4. The channel was 6m long, 6cm wide and 12cm deep. 100 larvae were introduced at the midpoint of the channel.

A5. The channel was 6m long, 6cm wide and 12cm deep. 100 larvae were introduced to one end of the channel.

Method B: Adapted from a study conducted concerning the post-feeding larval dispersal of blow flies (Lima et al. 2009). A rectangular arena, 2.5x1m was filled with soil, approximately 5cm deep. The soil used was taken from the Wildlife Garden at the Natural History Museum, London. Post-feeding larvae were introduced and left for one week. After 1 week the number of puparia recovered from each 10x10cm quadrant was recorded.

Experiment using Method B:

B1. 200 post feeding *Calliphora vicina* larvae were introduced to the centre of the arena.

Method C: Adapted from many studies examining the post feeding larval dispersal of blow flies (Gomes et al. 2006; Charabidze et al. 2008; Zimmer et al. 2010; Boulay et al. 2016). A circular arena, 2.5m diameter, was filled with sawdust approximately 5cm deep. Post-feeding larvae were introduced to the centre of the arena. After one week the number of puparia recovered from each 30x20cm quadrant was recorded.

Experiments using Method C:

C1. 1780 post feeding *Calliphora vicina* larvae were introduced to the centre of the arena.

Results

Figures 1, 2 and 3 are examples of the data produced by Methods A, B and C. Of most interest was the apparent aggregation of puparia, most clearly seen in Figures 1 and 3.

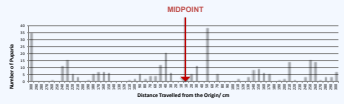


Figure 1. The distance dispersed from the midpoint by the larvae prior to pupariation in A3 (see Materials and Methods).

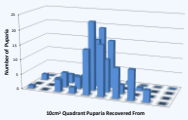


Figure 2. The number of puparia recovered from each 10x10cm quadrant in experiment B1.

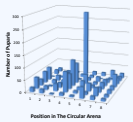


Figure 3. The number of puparia recovered from each 30x20cm quadrant in experiment C1.

To test the significance of these results, the data was analysed in frequency per sampling area.

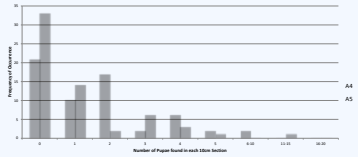


Figure 4. An example of the frequency of the number of puparia recovered from each section for experiments A4 and A5.

Aggregation was found to be highly significant in experiments A2, A3, A4, A5 and C1, $p<0.0001$ (χ^2 test) for all tests but A1.

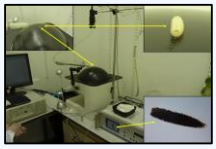
Discussion

Method A was considered best for determining distances travelled by larvae and Method C was most appropriate for measuring the distances travelled in conjunction with the directional preferences exhibited by the larvae. Method B was unsuitable as the arena was too small and had an uneven spread from the midpoint, and therefore did not have any advantages over Methods A or C.

These preliminary results provide evidence for aggregation of larvae for pupariation and this will be investigated further. Neighbourhood interactions of dispersing larvae and their perceived ability to detect other puparia in the pupariation substrate may be involved in the aggregation of puparia (Lima et al. 2009). However, other studies suggest that the larva's ability to detect neighbouring puparia and larvae in the pupariation substrate encourages them to move further to a less crowded section of substrate (Gomes & Zuben 2005).

Future Experiments

Other methods to be tested in the future will include the use of a SYNTECH ServoSphere. In response to larval movement detected by an overhead camera, the sphere rotates to maintain the larva in the camera's field of view at the top of the sphere. The movement of the sphere is recorded by the computer and, hence, enables the precise analysis of the movements of individual larvae (speed, direction and tortuosity).



Acknowledgements

Funding gratefully received from the Royal Entomological Society CPF (MMM), University of Winchester 175th Anniversary Fully Funded Studentship (MMM), University of Winchester RKE (APW), Natural History Museum (MJRH).

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Investigation of Post-Feeding Larval Dispersal in the UK Blow Fly: *Calliphora vicina* (Diptera: Calliphoridae)

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Abstract

In order to determine minPMI (minimum post mortem interval) from insect evidence it is necessary to examine the oldest specimens associated with the body and often these are not found on the body itself. Larval dispersal occurs after the feeding stage of the final, third instar. A greater understanding of this understudied stage is necessary to predict where dispersing larvae and the developing puparia are most likely to be found. Experiments were conducted looking at three factors of dispersal: the distance dispersed (horizontal dispersal), the depth dispersed (vertical dispersal) and whether the dispersing larvae aggregate prior to pupariation. Post-feeding *Calliphora vicina* larvae were introduced into different arenas to explore the different factors: a 600x10x10cm plastic arena was used to investigate horizontal dispersal and aggregation; a 60x8cm plastic pipe was used to look at vertical dispersal and aggregation; and a 100x10x10cm plastic arena was used to look at aggregation alone. The results suggest that over half of dispersing larvae burrow less than 20cm vertically, over half of dispersing larvae move less than 3m horizontally and that aggregation is occurring, producing a non-random, clumped abundance of puparia in the substrate.

Methodology

Experiments were conducted using 3 methods that were adapted from multiple studies looking at the post feeding larval dispersal of blow flies (Greenberg 1990; Andrade et al. 2002)

Figure 1 shows the 600x10x10cm plastic arena filled with topsoil. This arena was used to look at horizontal dispersal. After ten days the number of puparia recovered from each 10cm horizontal segment was recorded.

Figure 2 shows the 100x10x10cm plastic arenas filled with topsoil. These arenas were used to look at aggregation. After ten days the number of puparia recovered from each 10cm horizontal segment was recorded.

Figure 3 shows the 60x8cm plastic pipe filled with topsoil. This arena was used to look at vertical dispersal. After ten days the number of puparia recovered from each 3cm vertical segment was recorded.

Results

Figures 4, 5 and 6 are examples of the data produced using the three different methods described.

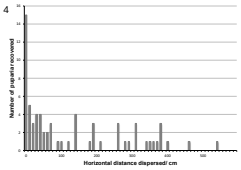


Figure 4 shows the data from one experiment looking at horizontal dispersal, the graph shows the number of puparia recovered from each 10cm section.

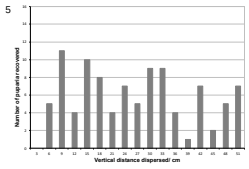


Figure 5 shows the data from one experiment looking at pre-puparial aggregation, the graph shows the number of puparia recovered from each 5cm section.

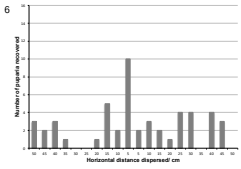


Figure 6 shows the data from one experiment looking at vertical dispersal, the graph shows the number of puparia recovered from each 3cm section.

For all 6m horizontal dispersal experiments, over half of the puparia were found in the first 3m, and the larvae dispersed the full 6m in only one experiment.

For all vertical dispersal experiments, over half of the puparia were found in the first 20cm, and the larvae never dispersed the full 60cm.

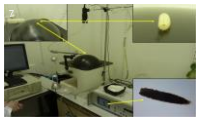
Aggregation was found to be highly significant in all experiments, $p<0.0001$ (χ^2 test), but for one of the 5 experiments that looked at vertical dispersal .

Discussion

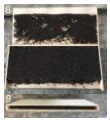
The results suggest that the majority of dispersing *Calliphora vicina* larvae do not disperse more than 3m horizontally or 20cm vertically. The results also strongly suggest that aggregation is occurring both horizontally and vertically. It is important to note that both the horizontal and vertical dispersion apparatus gave the dispersing larvae only one choice; to move either horizontally or vertically. Therefore experiments need to be conducted to determine how larval dispersal will be affected when they are given a choice. These experiments show that there may be predictability in the movement of dispersing larvae and that with more research we may be able to give crime scene officers advice about where there are most likely to find the dispersing larvae and puparia.

Future Experiments

Other methods to be tested in the future will include the use of a SYNTECH ServoSphere (Fig. 7.). In response to larval movement detected by an overhead camera, the sphere rotates to maintain the larva in the camera's field of view at the top of the sphere. The movement of the sphere is recorded by the computer and, hence, enables the precise analysis of the movements of individual larvae (speed, direction and tortuosity).



A rectangular, wooden apparatus (100x 50x5cm) was built to examine both horizontal and vertical dispersal simultaneously. Experiments conducted with this apparatus will help to build a picture of larval dispersal movements. Figure 8 shows the wooden apparatus open and figure 9 shows the apparatus from the top.



Acknowledgements

Funding gratefully received from the University of Winchester 175th Anniversary Fully Funded Studentship. The project is co-supervised by the University of Winchester and the Natural History Museum.

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